Integrin-linked kinase regulates vascular morphogenesis induced by vascular endothelial growth factor

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Summary

Integrin-linked kinase (ILK) is one of the signaling moieties that interact with the cytoplasmic domains of integrin β1 and β3 subunits. Integrin-mediated outside-in signals cooperate with vascular endothelial growth factor (VEGF) receptor to promote morphological changes, cell proliferation and motility in endothelial cells. In this report we demonstrate that VEGF-induced vessel morphogenesis of human umbilical vein endothelial cells (HUVEC) was inhibited by the transfection of a dominant negative, kinase-deficient ILK (ILK-KD), as well as by treatment with the phosphatidylinositol 3-kinase inhibitor LY294002. VEGF induced phosphorylation of protein kinase B (PKB/Akt), a regulator of cell survival and apoptosis, on serine 473, but not on threonine 308, in an ILK-dependent manner. Furthermore, transfection of antisense ILK (ILK-AS) blocked the survival effect of VEGF in annexin-V binding assays, and a VEGF-mediated decrease in caspase activity was reversed by both ILK-KD and ILK-AS as measured by a homogeneous caspase-3/7 assay. We also demonstrate that both chemotactic migration and cell proliferation of HUVEC induced by VEGF were suppressed by the inhibition of ILK. We conclude that ILK plays an important role in vascular morphogenesis mediated by VEGF.

Key words: HUVEC, ILK, Morphogenesis, PKB/Akt, Survival, VEGF

Introduction

Cell adhesion to the extracellular matrix (ECM) results in morphological changes, differentiation, proliferation, and the suppression of apoptosis (Damsky and Werb, 1992; Clark and Bruggs, 1995; Frisch and Ruoslahti, 1997; Schwartz, 1997). Adhesion molecules that are members of the integrin families mediate many of the cell-ECM interactions. Integrins contain a large extracellular domain that is responsible for ligand binding, a single transmembrane domain, and a cytoplasmic tail that in most cases consists of 20-70 amino acid residues (Hynes, 1992; Sastry and Horwitz, 1993). Integrin adhesion receptors are αβ heterodimeric transmembrane glycoproteins that interact with extracellular or cell surface molecules and with cytoplasmic molecules, including cytoskeletal and catalytic signaling proteins (Akiyama et al., 1990; Albelda and Buck, 1990; Schwartz et al., 1995). A select group of cytoplasmic proteins, which include catalytic proteins such as focal adhesion kinase (FAK) and noncatalytic proteins such as α-actinin, talin and paxillin are required to form focal adhesions (FA) in response to cell adhesion. Recent studies indicate that integrins not only receive signals from the ECM but also actively transmit 'inside-out' signals in promoting integrin ligand-binding affinity, assembly of ECM, and cell adhesion (Mosher et al., 1992; Wu et al., 1995; Ruoslahti, 1996).

The integrin-linked kinase (ILK) is a protein serine/threonine kinase that was initially identified in a yeast two-hybrid screen for integrin β1-tail-binding protein (Hannigan et al., 1996). It has been reported that ILK couples integrins to downstream signaling pathways involved in the suppression of apoptosis and in promoting cell cycle progression (Novak et al., 1998; Wu et al., 1998; Dedhar et al., 1999). At the molecular level, ILK consists of three structurally distinct motifs. At the N-terminal of ILK lie four ankyrin (ANK) repeats, which are responsible for binding to PINCH (Wu, 1999), which, in turn is required for localization of ILK to FA (Li et al., 1999). In addition, it connects ILK to other proteins, including Nck-2, a Src homology 3 (SH3)- and a SH2-containing adaptor protein, involved in the growth factor receptor kinase signaling pathways (Lehmann et al., 1990; Li et al., 1992; Meisenhelder and Hunter, 1992; Tu et al., 1998). C-terminal to the ANK repeats is a pleckstrin homology (PH)-like motif that probably binds a lipid product of phosphatidylinositol 3-kinase (PI 3-kinase) and participates in the regulation of the kinase activity (Alessi et al., 1997; Anderson et al., 1998). The ILK C-terminal domain exhibits significant homology to other kinase catalytic domains and can phosphorylate a number of protein substrates, such as a peptide derived from the β1 integrin cytoplasmic domain, the myelin basic protein, PKB/Akt and glycogen synthase kinase (GSK)-3 (Delcommenne et al., 1998).

It has been reported that the survival signal mediated by various growth factors and cytokines may be dependent on the PI 3-kinase/Akt signal transduction pathway (Minshall et al.,

1996; Yao and Cooper, 1996; Dudek et al., 1997; Franke et al., 1997; Kennedy et al., 1997; Songyang et al., 1997). The endothelial cell-specific mitogen, vascular endothelial growth factor (VEGF), has been shown to induce PI 3-kinase activity in a variety of endothelial cells and to be a key positive regulator of angiogenesis (Guo et al., 1995; Xia et al., 1996; Kevil et al., 1998). Additionally, it has been demonstrated that VEGF also promotes activation of PKB/Akt, FAK, mitogen-activated protein kinase (MAPK) and phospholipase Cγ (PLCγ) (Abedi and Zachary, 1997; Wu et al., 2000). Furthermore, integrins cooperate with VEGF receptor to promote the activation of an in vitro angiogenic program in endothelial cells (Byzova et al., 2000). However, so far a biological function for ILK in endothelial cells has not been demonstrated.

In this study we investigated the influence of a dominant negative, kinase-deficient ILK (ILK-KD) or antisense oligonucleotides against ILK (ILK-AS) on different steps of angiogenesis in human umbilical vein endothelial cells (HUVEC). We found that VEGF induced phosphorylation of PKB/Akt on serine 473, but not threonine 308, and the phosphorylation was suppressed by ILK-AS. Using several approaches, we demonstrate a critical role for ILK in endothelial cell function.

Materials and Methods

Cell culture and reagents

HUVEC, normal lung cells (CCD-8Lu), and normal hepatocytes (Cell System-Hc cells) were obtained from Cell Systems (Kirkland, WA) and were maintained in CS-C medium containing 10% fetal calf serum, according to the instructions of the supplier. Four human prostate cancer cell lines, DU-145, LNCaP, PC-3 and TSU-Pr1, were grown in RPMI 1640 supplemented with 10% fetal calf serum. All cells were passaged in 5% CO_2 at 37°C. Recombinant human vascular endothelial growth factor (rhVEGF) and recombinant human epidermal growth factor (EGF) were purchased from R&D Systems (Minneapolis, MN). Anti-PKB/Akt, anti-phospho-PKB/Akt (serine 473) and anti-phospho-PKB/Akt (threonine 308) were obtained from Cell Signaling Technology (Beverly, MA). Anti-VEGFR-2 and anti-phosphotyrosine (PY99) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Cellmatrix type I-A was from Nitta Gelatin Co. (Tokyo, Japan). LY294002, a selective phosphatidylinositol 3-kinase inhibitor, was obtained from Calbiochem (San Diego, CA).

Real time quantitative PCR

Isolation of total RNA was performed using TRIzol solution (Life Technologies Inc, Grand Island, NY). For reverse transcription, 1 μg of total RNA was converted into cDNA by AMV reverse transcriptase at 42°C for 1 hour in a 20 μl RT reaction. ILK mRNA was quantified by the dual-labeled fluorogenic probe methods. In brief, PCR amplification reaction mixtures (25 μl) contained cDNA, ILK forward primer (5′-TTT GCA GTG CTT CTG TGG GAA-3′), reverse primer (5′-CTA CTT GTC CTG CAT CTT CTC-3′), dual-labeled fluorogenic probe (5′-FAM TCA TGT GTG TAA GCT CAT GAA GAT CTG CAT TAMRA-3′), and TaqMan Universal PCR Master Mix (PE Applied Biosystems, Foster City, CA). Thermal cycle conditions included holding the reactions at 50°C for 2 minutes and 95°C for 10 minutes, and cycling for 40 cycles between 95°C for 15 seconds and 60°C for 1 minute. Results were collected and analyzed with an ABI Prism 7700 Sequence Detector System (PE Applied Biosystems). Final quantitation is reported as the absolute copy number of ILK transcripts per μg of total RNA.

cDNA vectors, site-directed mutagenesis and transfection

The CMV promoter-driven expression vectors containing full-length ILK wild-type (ILK-WT) cDNA or antisense ILK cDNA sequences were constructed using pcDNA3 expression vector (Invitrogen, Carlsbad, CA). Mutation was introduced into ILK-WT pcDNA3 using GeneEditor in vitro Site-Directed Mutagenesis System (Promega), according to the manufacturer's instructions. Mutant oligonucleotide (with the altered nucleotide underlined) was used to change glutamic acid at position 359 to a lysine (E359K, 5′-TGG GTA GCC CCC AAA GCT CTG CAG AAG-3′) within the kinase domain (Persad et al., 2001). The mutant clone was confirmed by sequencing. Transient transfections were performed with FuGENE6 Transfection Reagent (Roche Molecular Biochemicals) 18-24 hours before VEGF stimulation, according to the manufacturer's instructions.

Morphogenesis assay on collagen type I gel

Cellmatrix type I-A (150 μl) was added to the each well of 48-well culture plates and then incubated at 37°C for 1 hour to solidify the gel. HUVEC (1.5×10^4 cells) were transfected as above with empty vector or vector containing cDNA encoding ILK-KD, and then plated. 18 hours after plating, cells were stimulated with 30 ng/ml of rhVEGF and incubated at 37°C for 8 hours. The cells were stained with Giemsa dye and formation of the capillary-like tubes was evaluated by imaging system (KS400 ver. 2.0, Carl Zeiss Vision K. K.). Results are presented as the mean ± s.d. of eight fields derived from two wells.

Assay for cell adhesion to collagen type I

HUVEC were suspended in serum-free CS-C medium at 5.0×10^4 cells/well and incubated with or without rhVEGF for the indicated periods. The plates were washed with PBS twice, and 200 μl of medium containing WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)- 2*H*-tetrazolium•Na] was added to each well. The plates were read at a wavelength of 405 nm 2 hours after WST-1 addition. HUVEC adhesion was expressed as the mean ± s.d. of three wells.

Immunoblot analysis

Immunoblot analysis was performed as previously described (Basaki et al., 2002). Briefly, HUVEC stimulated with 30 ng/ml of rhVEGF were lysed in a lysis buffer (pH 7.4) containing 20 mM Tris-HCl, 1% Triton X-100, 50 mM each of NaCl and NaF, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin and leupeptin. The lysates were separated by SDS-PAGE, and then transferred to a nitrocellulose membrane. The membrane were incubated with the primary antibody and visualized with secondary antibody coupled to horseradish peroxidase (Cell Signaling Technology) and SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

ILK kinase assay

HUVEC stimulated with 30 ng/ml of rhVEGF were lysed in a lysis buffer. The lysates were precleaned with protein A-sepharose (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK), and then incubated with anti-ILK antibody (Upstate Biotechnology, Inc., Lake Placid, NY) at 4°C for 12 hours. After incubation, immune complexes were collected with protein A-sepharose. The immunoprecipitated ILK was incubated for 30 minutes at 30°C in a total volume of 50 μl of kinase reaction buffer (20 mM Hepes, pH 7.0, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 2 mM NaF, 1 mM Na_3VO_4, 100 μM ATP) in 96-well Reacti-Bind Metal™ Chelate Plate (Pierce) coated with his-tagged PKB/Akt, unactive (Upstate Biotechnology, Inc.). Phosphorylation of PKB/Akt was detected using anti-phospho-PKB/Akt (serine 473), secondary antibody coupled to horseradish

peroxidase (Cell Signaling Technology) and SuperSignal® ELISA Pico Chemiluminescent Substrate (Pierce). Chemiluminescence was measured using a WALLAC 1420 Multilabel Counter. Results are presented as the mean ± s.d. of four wells.

Analysis of apoptosis

For fluorescence-activated cell sorting analysis, cells were stained by fluorescein isothiocyanate-conjugated annexin-V. Annexin-V-positive cells are early apoptotic cells. Cells were cultured in 6-well plates for the indicated period with or without 30 ng/ml of rhVEGF. Immediately after harvest, cells were washed twice with PBS, and cells were stained using the annexin-V-FLUOS staining kit (Roche Molecular Biochemicals) according to the manufacturer's protocol. Analysis was carried out using a FACScan™ flow cytometer within 1 hour of staining. 30,000 events were analyzed using CELLQuest™ software (Becton Dickinson).

Caspase-3/7 assay

Caspase-3/7 activities were measured by Apo-ONE™ Homogeneous Caspase-3/7 Assay (Promega). Briefly, cells plated in 96-well plates at a density of 2.0×10^4 cells/well were lysed with an equal volume of lysis buffer containing caspase substrate, Z-DEVD-R100, and incubated at room temperature for 1 hour. The aliquots (150 μl) were transferred to white 96-well plates, and the fluorescence from each well was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a WALLAC 1420 Multilabel Counter. Results are presented as the mean ± s.d. of three wells.

Cell proliferation assay

Cell proliferation assay was carried out using a Cell Counting Kit (Wako Pure Chemical Industries ltd., Osaka, Japan). In brief, cells were plated in triplicate in 96-well plates at a density of 2.0×10^4 cells/well in CS-C medium. Following overnight culture, rhVEGF was added to a final concentration of 30 ng/ml, and the cells were incubated for 48 hours. After 48 hours, WST-1 was added and the cells were incubated for a further 3 hours. The plates were read at a wavelength of 405 nm using a Microplate Reader Model 3550 (Bio-Rad, Richmond, CA). Results are presented as the mean ± s.d. of three wells.

Chemotactic migration assay

Chemotactic migration of HUVEC was performed in a modified Boyden chamber using the method reported by Grotendorst et al. (Grotendorst et al., 1981). Briefly, the chemotactic response of the cells was assayed using 24-well chemotaxis chambers with upper and lower wells (Becton Dickinson) and 8 μm-pore size polycarbonate filters placed between wells. HUVEC (5×10^4 cells/200 μl) transfected with empty vector or vector containing cDNA-encoding ILK KD were applied to the top wells and incubated at 37°C for 18 hours in 5% CO_2. rhVEGF was added to the upper chamber and the migration was assessed by counting the number of cells that migrated to the lower side of the filter membrane after 5 hours incubation. Migrated cells were fixed with methanol and stained with Giemsa dye. The assays were performed in triplicate and the numbers of migrated cells were counted in seven fields per filter using the imaging system.

Results

Effect of ILK-KD on the vessel morphogenesis of endothelial cells in vitro

The expression of ILK has been demonstrated in various types of tumors, including prostate, breast, colorectal and lung, as well as in normal tissue (Ishii et al., 2001; Friedrich et al., 2002). Initially, we measured the expression levels of ILK mRNA in HUVEC. There were found to be 1.6×10^5 copies/μg total RNA (Table 1). Moreover, the differences in expression levels of the gene were minimal among cell lines tested, which included HUVEC, normal lung cells and normal hepatocytes, ranging from 1.6×10^5 to 2.8×10^5 copies/μg total RNA. In contrast, the expression levels of ILK mRNA in prostate cancer cells such as DU-145, LNCaP, PC-, and TSU-Pr1 were relatively higher than in HUVEC.

Table 1. Expression of ILK mRNA in various cells analyzed by real time quantitative PCR

Cells	Origin	ILK mRNA expression ($\times10^5$ copies/μg RNA)
HUVEC	Endothelial	1.60±0.45
CCD-8Lu	Normal lung	2.83±0.68
Cell System-Hc cells	Normal hepatocytes	1.80±0.38
DU-145	Prostate cancer	8.20±2.32
LNCaP	Prostate cancer	17.84±5.80
PC-3	Prostate cancer	9.10±1.92
TSU-Pr1	Prostate cancer	8.04±0.39

The quantification was based on the increased fluorescence detected by the ABI Prism 7700 sequence detection system resulting from hydrolysis of the target-specific probes by the 5′ nuclease activity of the AmpliTaq Gold during PCR amplification. Results are present as the mean±s.d.

Next, experiments were designed to evaluate whether VEGF-induced vessel morphogenesis in collagen type I gel was mediated by ILK. VEGF induced formation of capillary-like tubes in a concentration-dependent fashion, from 10 to 100 ng/ml, in HUVEC (Fig. 1A). Although tube length was not significantly affected by the transfection of an empty vector, VEGF-triggered capillary-like tube formation was abrogated by ILK-KD. Additionally, treatment with LY294002 (10 μM) strongly inhibited tube formation (Fig. 1B).

It has been reported that one of the central systems in capillary formation is the adhesion of cells to extracellular matrix (ECM) via integrin/ECM binding (Gamble et al., 1993; Beck and D'Amore, 1997). To determine whether ILK is involved in VEGF-induced increases in cell adhesion, we assessed the effect of ILK-KD on HUVEC adhesion to collagen type I. The addition of VEGF increased cell attachment in a concentration-dependent manner from 10 to 100 ng/ml in HUVEC (Fig. 2A), so the inhibitory activity of ILK-KD and LY294002 on cell adhesion was tested with a VEGF concentration of 30 ng/ml. Although VEGF-enhanced cell adhesion was not significantly affected by the transfection of an empty vector, cell adhesion was partially suppressed by ILK-KD to 53.0%, compared with the VEGF-treated control. Additionally, treatment with LY294002 (10 μM) also inhibited adhesion to 55.0% of the control (Fig. 2B). These results indicate that ILK and PI 3-kinase play important roles in the endothelial cell morphogenesis induced by VEGF stimulation.

Phosphorylation of Akt is abrogated by ILK inhibition in HUVEC

It has been reported that activation of PKB/Akt is mediated in several growth factor signaling pathways. Experiments were designed to evaluate whether VEGF-induced PKB/Akt

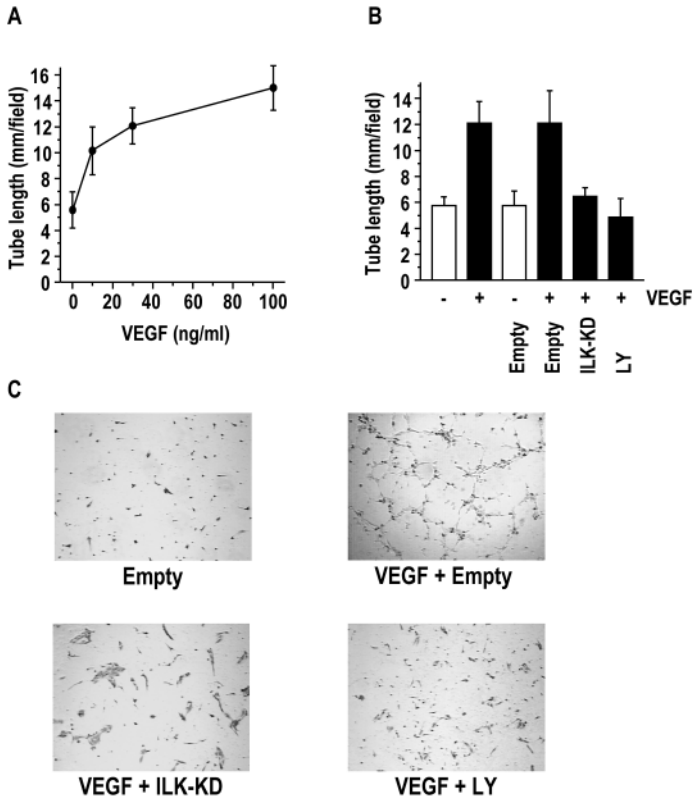

Fig. 1. Effects of ILK-KD and LY294002 on morphological changes induced by VEGF. (A) HUVEC (1.5×10^4 cells/well) were incubated for 8 hours in serum-free medium in the presence of the indicated concentration of rhVEGF. The morphological changes of HUVEC on collagen type I gel were evaluated as described in Materials and Methods. (B) HUVEC were transiently transfected with the indicated plasmids 18 hours before VEGF stimulation. LY294002 was added 30 minutes before the stimulation. The cells were incubated in serum-free medium in the presence of 30 ng/ml rhVEGF and the morphological changes were examined. Data are expressed as the mean ± s.d. (C) Images showing typical morphological changes of HUVEC.

phosphorylation was mediated by ILK. HUVEC were incubated in the absence or presence of VEGF, and phosphorylation of PKB/Akt was detected by immunoblot analysis using anti-phospho-PKB/Akt (serine 473) and anti-phospho-PKB/Akt (threonine 308). As shown in Fig. 3A, VEGF induced phosphorylation of PKB/Akt on serine 473, but not on the threonine 308 residue in HUVEC. To determine the involvement of ILK on this serine 473 phosphorylation, we performed transient transfections with vectors containing ILK-KD or antisense ILK (ILK-AS). ILK-AS suppressed VEGF-induced PKB/Akt phosphorylation on the serine 473 residue with reduction of expression level of ILK protein (Fig. 3B). ILK-KD also inhibited the serine 473 phosphorylation of PKB/Akt.

It has been suggested, from several studies, that ILK may not be a bona-fide protein kinase for PKB/Akt, or any other substrate. We therefore examined whether VEGF increases ILK kinase activity, phosphorylating PKB/Akt in HUVEC, by using his-tagged PKB/Akt as a substrate. As shown in Fig. 3C, maximal activation of ILK kinase activity in the cells occurred 5 minutes after VEGF stimulation, and then rapidly disappeared. ILK-KD could behave as a dominant negative, because ILK kinase activity and serine 473 phosphorylation of PKB/Akt were suppressed by ILK-KD. Moreover, the kinase activity was prevented by ILK-AS (Fig. 3D). It is well known that activated VEGFR associates with adaptor molecules including Nck, Grb2 and PI 3-kinase. We examined whether ILK is able to associates with the VEGFR complex in VEGF-stimulated HUVEC. The experimental data shown in Fig. 4A demonstrate that VEGF induced ILK recruitment to the VEGFR complex. In addition, ILK kinase activity was inhibited by treatment with 10 μM LY294002 (Fig. 4B). These data indicated that the PKB/Akt serine 473 phosphorylation step in the VEGF signaling pathway was mediated by ILK protein kinase.

ILK mediates survival signaling of VEGF

PKB/Akt signaling has been shown to mediate anti-apoptotic activity in various tumor cells. To determine whether ILK mediates a survival signal initiated by VEGF, we examined the survival of VEGF-treated HUVEC by measuring their annexin-V binding activity. The cells were cultured in the absence or

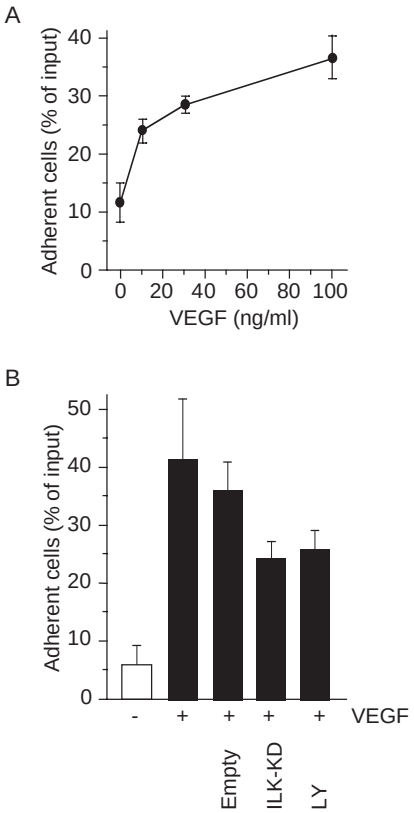

Fig. 2. VEGF increased adhesion of HUVEC to collagen type I. (A) HUVEC were starved for 18 hours and then plated on 96-well plates (5.0×10^4 cells/well). VEGF was added to the medium 10 minutes before plating and the adherent cells were measured by WST-1 assay, as described in Materials and Methods. (B) HUVEC were transiently transfected with the indicated plasmids 18 hours before VEGF stimulation. LY294002 was added 30 minutes before the stimulation. Data are expressed as the mean ± s.d.

the presence of 30 ng/ml VEGF for 48 hours and their annexin-V binding activity analyzed using flow cytometry (Fig. 5). The percentage of annexin-V^+ cells reached 45.1% in the non-treated HUVEC, whereas only 13.3% of the cells were labeled with annexin-V when the cells were treated with VEGF. To demonstrate the involvement of ILK in the anti-apoptotic effect of VEGF, ILK was specifically inhibited by the expression of ILK-AS. Of the cells transfected with ILK-AS vector, 45.9% were positive for annexin-V binding, thus indicating that ILK is involved in the apoptosis-suppressing effect of VEGF.

Caspase activity is known to increase during the early stage of apoptosis (Salvesen and Dixit, 1996; Sgorbissa et al., 1999). To further our investigation, the cells were cultured in the absence or the presence of 30 ng/ml VEGF for 48 hours and caspase-3/7 activity was measured. As shown in Fig. 6, activation of caspase-3/7 was induced in HUVEC by serum starvation, and this activation was suppressed to 37.8% by the addition of VEGF. Inhibition of ILK by transfection of the vector containing ILK-KD or ILK-AS reversed the effect of

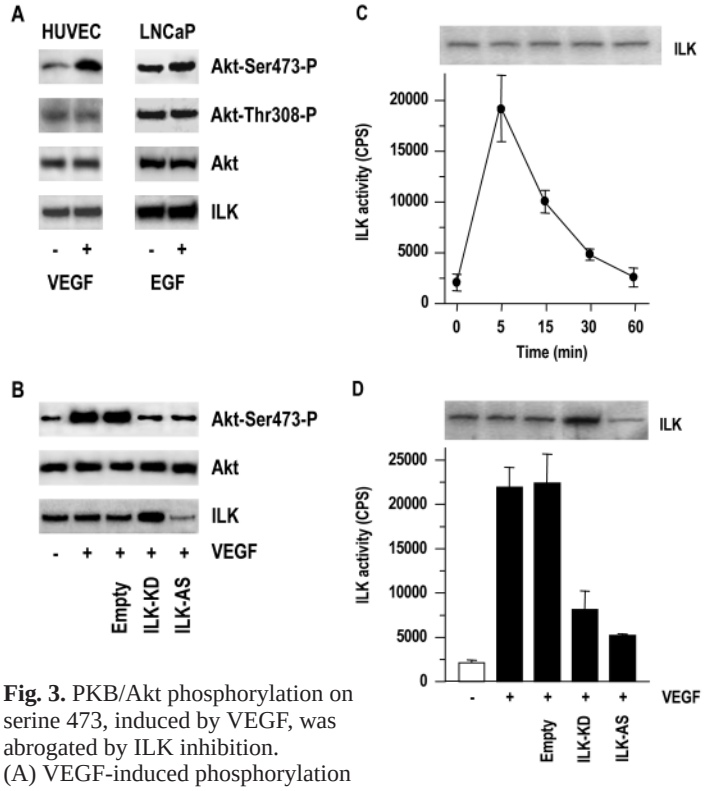

Fig. 3. PKB/Akt phosphorylation on serine 473, induced by VEGF, was abrogated by ILK inhibition. (A) VEGF-induced phosphorylation of PKB/Akt. HUVEC and LNCaP were stimulated with 30 ng/ml of rhVEGF and 100 ng/ml of rhEGF, respectively, for 15 minutes. (B) Inhibition of serine 473 phosphorylation of PKB/Akt by ILK-KD or ILK-AS. The cells transfected with the indicated plasmids were stimulated with 30 ng/ml of rhVEGF for 15 minutes, and then PKB/Akt phosphorylation on serine 473 was detected by immunoblot analysis. (C) VEGF increased kinase activity of ILK. The cells were lysed, and ILK was immunoprecipitated from cell extracts. ILK activity was determined using PKB/Akt as a substrate. Anti-ILK immunoblot was prepared from the same immunoprecipitates used for the kinase assay (top panel). (D) Inhibition of kinase activity of ILK by ILK-KD or ILK-AS. The cells transfected with the indicated plasmids were stimulated with 30 ng/ml of rhVEGF for 5 minutes, and then ILK activity was measure by ILK kinase assay. Anti-ILK immunoblot was prepared from the same immunoprecipitates used for the kinase assay (top panel).

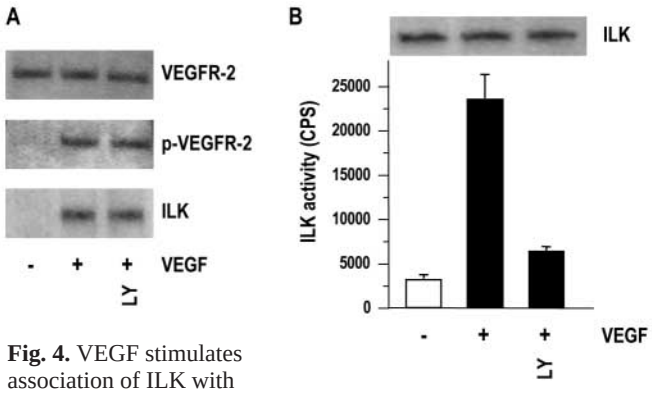

Fig. 4. VEGF stimulates association of ILK with VEGFR complex. (A) VEGF stimulates recruitment of ILK to VEGFR-2. The cells were cultured in the presence or absence of 30 ng/ml of rhVEGF for 5 minutes. The cells were lysed, and anti-VEGFR-2 immunoprecipitates were prepared and immunoblotted with anti-VEGFR-2, anti-phosphotyrosine or anti-ILK antibodies. (B) Inhibition of kinase activity of ILK by LY294002. The cells were stimulated with 30 ng/ml of rhVEGF for 5 minutes, and then ILK activity was measure by ILK kinase assay. LY294002 was added 30 minutes before the VEGF stimulation. Anti-ILK immunoblot was prepared from same immunoprecipitates used for the kinase assay (top panel).

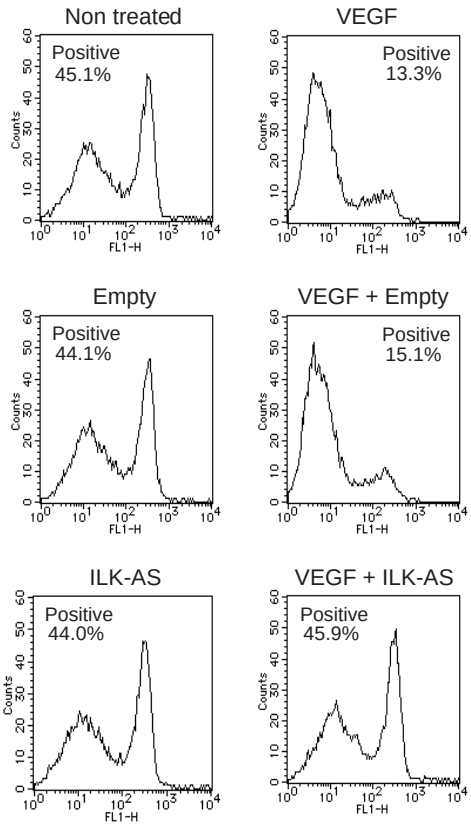

Fig. 5. Effect of ILK-AS on the apoptosis-suppressive activity of VEGF. HUVEC were transiently transfected with the indicated plasmids and incubated for 24 hours. Apoptosis was then induced by serum starvation in the presence or absence of 30 ng/ml of rhVEGF for 48 hours. The cells were then harvested, and apoptotic cell death was determined by flow cytometry analysis using annexin-V-fluorescein. One of two comparable experiments is shown.

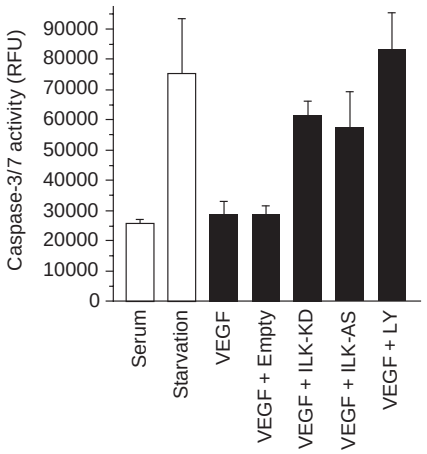

Fig. 6. Effect of ILK-KD and ILK-AS on VEGF-mediated suppression of caspase-3/7 activities. HUVEC were transiently transfected with the indicated plasmids and incubated for 24 hours. Caspase-3/7 activation was then induced by serum starvation in the presence or absence of 30 ng/ml of rhVEGF for 48 hours. Caspase-3/7 activities were measured by Apo-ONE^{TM} Homogeneous caspase-3/7 assay, as described in Materials and Methods.

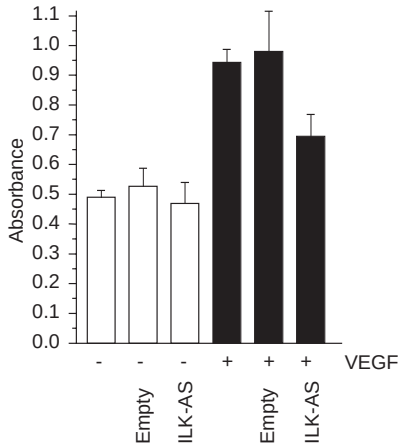

Fig. 7. Effect of ILK-AS on HUVEC proliferation stimulated with VEGF. HUVEC were transiently transfected with the indicated plasmids and incubated for 16 hours. The cells were cultured in the presence or absence of 30 ng/ml of rhVEGF for 48 hours. Viable cells were measured by WST assay. Data are expressed as the mean ± s.d.

VEGF, resulting in an increase in active caspase-3/7 levels, while caspase-3/7 activities of empty vector-treated cells were similar to the activities of VEGF-treated controls. Furthermore, treatment with 10 μM LY294002 also reversed the VEGF-mediated decrease in caspase activities. These data indicate that the survival effect of VEGF was mediated by the inhibition of caspase-3/7 activity via ILK signaling.

ILK is involved in proliferation induced by VEGF

A number of studies have demonstrated that VEGF is a potent angiogenic factor inducing proliferation, chemotactic migration, and morphological changes in vascular endothelial cells (Guo et al., 1995; Xia et al., 1996; Kevil et al., 1998; Basaki et al., 2001). We further investigated the role of ILK in HUVEC proliferation induced by VEGF. The cells were cultured in the absence or presence of VEGF for 48 hours, and viable cells were measured by WST assay. As shown in a representative experiment in Fig. 7, VEGF induced a proliferative response of approximately 1.6-fold in HUVEC. VEGF-induced proliferation was suppressed by transfection of ILK-AS. In contrast, in the absence of VEGF, the expression of antisense ILK did not affect the surviving cells.

VEGF-driven chemotactic migration is suppressed by ILK-KD

The migration of HUVEC was studied using a modified Boyden chamber method, in which the endothelial cells migrate through a porous membrane toward a stimulus, in this case VEGF. We investigated the inhibitory activity of ILK-KD and LY294002 on the migration of cells placed in the upper part of the chamber, with the lower part of the Boyden chamber containing medium supplemented with 30 ng/ml of VEGF. The results of this experiment are shown in Fig. 8. The migration of HUVEC stimulated with VEGF was increased compared to

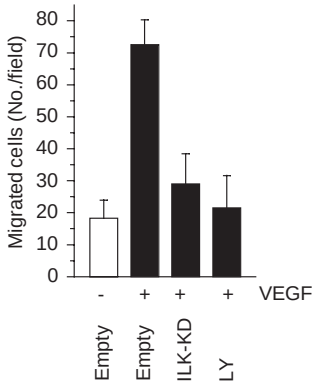

Fig. 8. Effects of ILK-KD and LY294002 on chemotactic migration of HUVEC stimulated by VEGF. HUVEC were transiently transfected with the indicated plasmids 18 hours before VEGF stimulation. LY294002 was added to the upper chamber containing HUVEC 30 minutes before the stimulation. rhVEGF was added to the lower chamber at a concentration of 30 ng/ml and migratory activity of the cells was estimated, based on the number of cells migrating to the lower chamber. Data are expressed as the mean ± s.d.

control medium (53.7±8.3 cells/field). Transfection of ILK-KD vector decreased migration by 82.2% compared with the control. Furthermore, treatment with 10 μM LY294002 strongly inhibited HUVEC migration. These data imply that ILK was involved in the VEGF signal transduction mediating the chemotactic migration.

Discussion

ILK is a serine/threonine protein kinase that interacts with the β1 or β3 integrin subunit via the carboxyl terminal region of its kinase catalytic domain, thereby mediating integrin signal transduction. Various integrin signaling pathways collaborate with VEGF receptor signaling in the activation of an in vitro

angiogenic program in endothelial cells. However, the signal transduction cascades, which connect VEGF receptor signaling and integrin signaling, are incompletely characterized. In HUVEC, tyrosine phosphorylation of VEGFR-2 is induced by the binding of its soluble ligand VEGF. Using these cells, we have demonstrated that ILK-KD or ILK-AS inhibit different steps of VEGF-induced angiogenesis.

The stimulation of endothelial cells by various growth factors, such as VEGF or fibroblast growth factor-2, is required for the multi-step process of angiogenesis (Cronauer et al., 1997; Kumar et al., 1998; Shibusa et al., 1998). VEGFR-2 is a receptor tyrosine kinase, which is responsible for the angiogenic activity of VEGF. The binding of VEGF to this receptor leads to its activation through the phosphorylation of a tyrosine residue (Heldin, 1995). Activated VEGFR-2 associates with adaptor molecules such as insulin receptor substrate-1, Shc, Grb2, Nck, PLCγ and SHP-1, and the formed transductsome mediates the activation of PI 3-kinase, MAPK, Jun-N-terminal kinase (JNK) and FAK (Ellis et al., 1990; Terman et al., 1991; Settleman et al., 1992; Waltenberger et al., 1994; Seetharam et al., 1995; Takahashi and Shibuya, 1997). However, VEGF-driven, capillary-like tube formation in HUVEC was not affected by PD98059 or SB203580, selective inhibitors of Erk and p38MAPK, respectively (data not shown). Morphological changes of HUVEC, induced by VEGF, were inhibited by ILK-KD as well as by LY294002, a selective inhibitor of PI 3-kinase. Our data indicated the involvement of PI 3-kinase in the VEGF signaling pathway, mediating morphological changes in HUVEC. PI 3-kinase phosphorylates specific phosphoinositides to generate the lipids PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. These phosphorylated products bind to PH domains of downstream effector enzymes such as phosphoinositide-dependent kinase-1, PKB/Akt and the ζ-type protein kinase C (Nakanishi et al., 1993; Wymann and Pirola, 1998; Standaert et al., 2001). Furthermore, ILK activity is stimulated in a PI 3-kinase-dependent fashion by the binding of PtdIns(3,4,5)P_3 to the PH-like motif at the carboxyl-terminal of the ANK repeat of ILK (Dedhar, 2000). We show for the first time that the VEGF-induced morphological changes in HUVEC are inhibited by ILK-KD, which is a mutant form with glutamic acid 359 in the kinase domain replaced by a lysine. The mutant functions as a potent dominant negative (Huang et al., 2000; Somasiri et al., 2001). Therefore, we conclude that PI 3-kinase/ILK signaling is important in the morphological changes of endothelial cells.

ILK fulfills many of the properties of an upstream regulator that phosphorylates PKB/Akt and GSK-3β. The phosphorylation of PKB/Akt on the serine 473 residue is highly inducible by a number of different growth factors, whereas threonine 308 phosphorylation is largely constitutive (Alessi et al., 1997; Downward, 1998). We confirmed that VEGF stimulation induced phosphorylation of serine 473 but not threonine 308 in HUVEC. In addition, this phosphorylation was suppressed by transfection of antisense ILK or ILK-KD. These studies are in agreement with findings that ILK phosphorylates PKB/Akt on serine 473 in growth factor signaling pathways. In our apoptosis assay, we found decreased levels of VEGF-dependent survival activities when the cells were transfected with ILK-AS. It has previously been demonstrated in fibroblasts, neuronal cells, and others that a variety of growth factors rapidly activate PKB/Akt via PI 3-kinase activation, and that activated PKB/Akt promotes survival. VEGF also exhibits anti-apoptotic activity through the phosphorylation and activation of PKB/Akt in endothelial cells. PKB/Akt increases survival through several pathways, resulting in the downstream inhibition of processing and activation of caspases (Barber et al., 2001; Chang et al., 2001; Stratford et al., 2001). In homogeneous caspase-3/7 assays, we showed that the activities of caspases were decreased by VEGF stimulation, and that ILK inhibition by transfection of ILK-KD or ILK-AS reversed this decrease. Additionally, we demonstrated that LY294002 also reversed the decrease in caspase activities. The current studies suggest that ILK may function as a component of a signal transduction pathway regulating endothelial cell survival mediated by PKB/Akt.

In addition to the morphological changes of endothelial cells, different steps such as cell adhesion to ECM, cell proliferation, and chemotactic migration are involved in the angiogenesis program. These steps are promoted by the stimulation of various angiogenic factors, including VEGF. We report that VEGF enhanced the attachment of HUVEC to collagen type I in a concentration-dependent manner, and this attachment was suppressed by the transfection of ILK-KD. ILK is located in the focal adhesion area and functions in integrin-mediated cell adhesion through an association with cytoskeletal components (Nikolopoulos and Turner, 2001; Yamaji et al., 2001; Tu et al., 2001). In endothelial cells, as well as other various types of cells, integrins play a central role in cell attachment to ECM, and mediate signal transduction through the cell membrane in both directions: 'outside-in signaling' and 'inside-out signaling'. The binding of integrins to ligands regulates cellular functions through associations between integrin β tails and binding partners such as ILK, talin, α-actinin, FAK, paxillin, Grb2 and Shc (Schaller et al., 1995; Law et al., 1996). We conclude that ILK may partially function in the cell-ECM adhesion of endothelial cells promoted by VEGF. Both proliferation and migration of endothelial cells increased by growth factors are also important phenomena in angiogenesis. Actually, it has already been reported that these biological responses were promoted by VEGF, one of the angiogenesis molecules, in endothelial cells. Two major signaling cascades, PI 3-kinase and MAPK, known to mediate proliferation and migration, are activated by VEGF (Gerber et al., 1998; Doanes et al., 1999; Takahashi et al., 1999). Since ILK-KD suppressed both proliferation and chemotactic migration promoted by VEGF, the PI 3-kinase/ILK/Akt pathway may support migration in endothelial cells. The MAPK pathway did not play a role in HUVEC migration, since PD98059 did not block cell migration (data not shown). It should be noted that ILK has the ability to support both chemotactic migration and cell proliferation mediated by VEGF in endothelial cells.

In summary, our findings provide evidence that VEGF-induced morphogenesis in endothelial cells is mediated by PI 3-kinase/ILK signal transduction pathways. Moreover, we have demonstrated a role for ILK in VEGF survival signals through the inhibition of caspase activation. Further examination of this ILK signaling pathway may yield targets for anticancer therapy aimed at inducing blood vessel regression and endothelial cell apoptosis.

References

Abedi, H. and Zachary, I. (1997). Vascular endothelial growth factor stimulates tyrosine phosphorylation and recruitment to new focal adhesions of focal adhesion kinase and paxillin in endothelial cells. *J. Biol. Chem*. **272**, 15442-15451.

Akiyama, S. K., Nagata, K. and Yamada, K. M. (1990). Cell surface receptors for extracellular matrix components. *Biochim. Biophy. Acta* **1031**, 91-110.

Albelda, S. M. and Buck, C. A. (1990). Integrins and other cell adhesion molecules. *FASEB J*. **4**, 2868-2880.

Alessi, D. R., Deak, M., Casamayor, A., Caudwell, F. B., Morrice, N., Norman, D. G., Gaffney, P., Reese, C. B., MacDougall, C. N., Harbison, D. et al. (1997). 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the Drosophila DSTPK61 kinase. *Curr. Biol*. **7**, 776-789.

Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B. and Cohen, P. (1997). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase B alpha. *Curr. Biol*. **7**, 261-269.

Anderson, K. E., Coadwell, J., Stephens, L. R. and Hawkins, P. T. (1998). Translocation of PDK-1 to the plasma membrane is important in allowing PDK-1 to activate protein kinase B. *Curr. Biol*. **8**, 684-691.

Barber, A. J., Nakamura, M., Wolpert, E. B., Reiter, C. E., Seigel, G. M., Antonetti, D. A. and Gardner, T. W. (2001). Insulin rescues retinal neurons from apoptosis by a phosphatidylinositol 3-kinase/Akt-mediated mechanism that reduces the activation of caspase-3. *J. Biol. Chem*. **276**, 32814-32821.

Basaki, Y., Chikahisa, L., Aoyagi, K., Miyadera, K., Yonekura, K., Hashimoto, A., Okabe, S., Wierzba, K. and Yamada, Y. (2001). Gamma-hydroxybutyric acid and 5-fluorouracil, metabolites of UFT, inhibit the angiogenesis induced by vascular endothelial growth factor. *Angiogenesis* **4**, 163-173.

Basaki, Y., Ikizawa, K., Kajiwara, K. and Yanagihara, Y. (2002). CD40-mediated tumor necrosis factor receptor-associated factor 3 signaling upregulates IL-4-induced germline C epsilon transcription in a human B cell line. *Arch. Biochem. Biophys*. **405**, 199-204.

Beck, L., Jr and D'Amore, P. A. (1997). Vascular development: cellular and molecular regulation. *FASEB J*. **11**, 365-373.

Byzova, T. V., Goldman, C. K., Pampori, N., Thomas, K. A., Bett, A., Shattil, S. J. and Plow, E. F. (2000). A mechanism for modulation of cellular responses to VEGF: activation of the integrins. *Mol. Cell* **6**, 851-860.

Chang, H. C., Tsai, L. H., Chuang, L. Y. and Hung, W. C. (2001). Role of AKT kinase in sphingosine-induced apoptosis in human hepatoma cells. *J. Cell Physiol*. **188**, 188-193.

Clark, E. A. and Brugge, J. S. (1995). Integrins and signal transduction pathways: the road taken. *Science* **268**, 223-239.

Cronauer, M. V., Hittmair, A., Eder, I. E., Hobisch, A., Culig, Z., Ramoner, R., Zhang, J., Bartsch, G., Reissigl, A., Radmayr, C. et al. (1997). Basic fibroblast growth factor levels in cancer cells and in sera of patients suffering from proliferative disorders of the prostate. *Prostate* **31**, 223-233.

Damsky, C. H. and Werb, Z. (1992). Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. *Curr. Opin. Cell Biol*. **4**, 772-781.

Dedhar, S. (2000). Cell-substrate interactions and signaling through ILK. *Curr. Opin. Cell Biol*. **12**, 250-256.

Dedhar, S., Williams, B. and Hannigan, G. (1999). Integrin linked kinase (ILK): a regulator of integrin and growth-factor signaling. *Trends Cell Biol*. **9**, 319-323.

Delcommenne, M., Tan, C., Gray, V., Rue, L., Woodgett, J. and Dedhar, S. (1998). Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. *Proc. Natl. Acad. Sci. USA* **95**, 11211-11216.

Doanes, A. M., Hegland, D. D., Sethi, R., Kovesdi, I., Bruder, J. T. and Finkel, T. (1999). VEGF stimulates MAPK through a pathway that is unique for receptor tyrosine kinases. *Biochem. Biophys. Res. Commun.* **255**, 545-548.

Downward, J. (1998). Mechanisms and consequences of activation of protein kinase B/Akt. *Curr. Opin. Cell Biol.* **10**, 262-267.

Dudek, H., Datta, S. R., Franke, T. F., Birnbaum, M. J., Yao, R., Cooper, G. M., Segal, R. A., Kaplan, D. R. and Greenberg, M. E. (1997). Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science* **275**, 661-665.

Ellis, C., Moran, M., McCormick, F. and Pawson, T. (1990). Phosphorylation of GAP and GAP-associated proteins by transforming and mitogenic tyrosine kinases. *Nature* **343**, 377-381.

Franke, T. F., Kaplan, D. R., Cantley, L. C. and Toker, A. (1997). Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. *Science* **275**, 665-668.

Friedrich, E. B., Sinha, S., Li, L., Dedhar, S., Force, T., Rosenzweig, A. and Gerszten, R. E. (2002). Role of integrin-linked kinase in leukocyte recruitment. *J. Biol. Chem*. **277**, 16371-16375.

Frisch, S. M. and Ruoslahti, E. (1997). Integrins and anoikis. *Curr. Opin. Cell Biol*. **9**, 701-706.

Gamble, J. R., Matthias, L. J., Meyer, G., Kaur, P., Russ, G., Faull, R., Berndt, M. C. and Vadas, M. A. (1993). Regulation of in vitro capillary tube formation by anti-integrin antibodies. *J. Cell Biol.* **121**, 931-943.

Gerber, H. P., McMurtrey, A., Kowalski, J., Yan, M., Keyt, B. A., Dixit, V. and Ferrara, N. (1998). Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J. Biol. Chem*. **273**, 30336-30343.

Grotendorst, G. R., Seppa, H. E., Kleinman, H. K. and Martin, G. R. (1981). Attachment of smooth muscle cells to collagen and their migration toward platelet-derived growth factor. *Proc. Natl. Acad. Sci. USA* **78**, 3669-3672.

Guo, D., Jia, Q., Song, H. Y., Warren, R. S. and Donner, D. B. (1995). Vascular endothelial cell growth factor promotes tyrosine phosphorylation of mediators of signal transduction that contain SH2 domains: Association with endothelial cell proliferation. *J. Biol. Chem*. **270**, 6729-6739.

Hannigan, G. E., Leung-Hagesteijn, C., Fitz-Gibbon, L., Coppolino, M. G., Radeva, G., Filmus, J., Bell, J. C, and Dedhar, S. (1996). Regulation of cell adhesion and anchorage-dependent growth by a new beta 1-integrin-linked protein kinase. *Nature* **379**, 91-96.

Heldin, C. H. (1995). Dimerization of cell surface receptors in signal transduction. *Cell*. **80**, 213-223.

Huang, Y., Li, J., Zhang, Y. and Wu, C. (2000). The roles of integrin-linked kinase in the regulation of myogenic differentiation. *J. Cell Biol*. **150**, 861-872.

Hynes, R. O. (1992). Integrins: Versatility, modulation, and signalling in cell adhesion. *Cell* **69**, 11-25.

Ishii, T., Satoh, E. and Nishimura, M. (2001). Integrin-linked kinase controls neurite outgrowth in N1E-115 neuroblastoma cells. *J. Biol. Chem*. **276**, 42994-43003.

Kennedy, S. G., Wagner, A. J., Conzen, S. D., Jordan, J., Bellacosa, A., Tsichlis, P. N. and Hay, N. (1997). The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. *Genes Dev*. **11**, 701-713.

Kevil, C. G., Payne, D. K., Mire, E. and Alexander, J. S. (1998). Vascular permeability factor/vascular endothelial cell growth factor-mediated permeability occurs through disorganization of endothelial junctional proteins. *J. Biol. Chem.* **273**, 15099-15103.

Kumar, R., Yoneda, J., Bucana, C. D. and Fidler, I. J. (1998). Regulation of distinct steps of angiogenesis by different angiogenic molecules. *Int. J. Oncol*. **12**, 749-757.

Law, D. A., Nannizzi-Alaimo, L. and Phillips, D. R. (1996). Outside-in integrin signal transduction. Alpha IIb beta 3-(GP IIb IIIa) tyrosine phosphorylation induced by platelet aggregation. *J. Biol. Chem*. **271**, 10811-10815.

Lehmann, J. M., Riethmuller, G. and Johnson, J. P. (1990). Nck, a melanoma cDNA encoding a cytoplasmic protein consisting of the src homology units SH2 and SH3. *Nucleic Acids Res*. **18**, 1048.

Li, F., Zhang, Y. and Wu, C. (1999). Integrin-linked kinase is localized to cell-matrix focal adhesions but not cell-cell adhesion sites and the focal adhesion localization of integrin-linked kinase is regulated by the PINCH-binding ANK repeats. *J. Cell Sci*. **112**, 4589-4599.

Li, W., Hu, P., Skolnik, E. Y., Ullrich, A. and Schlessinger, J. (1992). The SH2 and SH3 domain-containing Nck protein is oncogenic and a common target for phosphorylation by different surface receptors. *Mol. Cell. Biol*. **12**, 5824-5833.

Meisenhelder, J. and Hunter, T. (1992). The SH2/SH3 domain-containing protein Nck is recognized by certain anti-phospholipase C-gamma 1 monoclonal antibodies, and its phosphorylation on tyrosine is stimulated by platelet-derived growth factor and epidermal growth factor treatment. *Mol. Cell. Biol*. **12**, 5843-5856.

Minshall, C., Arkins, S., Freund, G. G. and Kelley, K. W. (1996). Requirement for phosphatidylinositol 3′-kinase to protect hemopoietic progenitors against apoptosis depends upon the extracellular survival factor. *J. Immunol*. **156**, 939-947.

Mosher, D. F., Sottile, J., Wu, C. and McDonald, J. A. (1992). Assembly of extracellular matrix. *Curr. Opin. Cell Biol.* **4**, 810-818.
Nakanishi, H., Brewer, K. A. and Exton, J. H. (1993). Activation of the zeta isozyme of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem*. **268**, 13-16.
Nikolopoulos, S. N. and Turner, C. E. (2001). Integrin-linked kinase (ILK) binding to paxillin LD1 motif regulates ILK localization to focal adhesions. *J. Biol. Chem*. **276**, 23499-23505.
Novak, A., Hsu, S. C., Leung-Hagesteijn, C., Radeva, G., Papkoff, J., Montesano, R., Roskelley, C., Grosschedl, R. and Dedhar, S. (1998). Cell adhesion and the integrin-linked kinase regulate the LEF-1 and beta-catenin signaling pathways. *Proc. Natl. Acad. Sci. USA* **95**, 4374-4379.
Persad, S., Attwell, S., Gray, V., Mawji, N., Deng, J. T., Leung, D., Yan, J., Sanghera, J., Walsh, M. P. and Dedhar, S. (2001). Regulation of protein kinase B/Akt-serine 473 phosphorylation by integrin-linked kinase: critical roles for kinase activity and amino acids arginine 211 and serine 343. *J. Biol. Chem*. **276**, 27462-27469.
Ruoslahti, E. (1996). Integrin signaling and matrix assembly. *Tumour Biol.* **17**, 117-124.
Salvesen, G. S. and Dixit, V. M. (1996). Caspase activation: the induced-proximity model. *Proc. Natl. Acad. Sci. USA* **96**, 10964-10967.
Sastry, S. K. and Horwitz, A. F. (1993). Integrin cytoplasmic domains: mediators of cytoskeletal linkages and extra-and intracellular initiated transmembrane signaling. *Curr. Opin. Cell Biol*. **5**, 819-831.
Schaller, M. D., Otey, C. A., Hildebrand, J. D. and Parsons, J. T. (1995). Focal adhesion kinase and paxillin bind to peptides mimicking beta integrin cytoplasmic domains. *J. Cell Biol*. **130**, 1181-1187.
Schwartz, M. A. (1997). Integrins, oncogenes, and anchorage independence. *J. Cell Biol*. **139**, 575-578.
Schwartz, M. A., Schaller, M. D. and Ginsberg, M. H. (1995). Integrins: emerging paradigms of signal transduction. *Annu. Rev. Cell Dev. Biol.* **11**, 549-599.
Seetharam, L., Gotoh, N., Maru, Y., Neufeld, G., Yamaguchi, S. and Shibuya, M. (1995). A unique signal transduction from FLT tyrosine kinase, a receptor for vascular endothelial growth factor VEGF. *Oncogene* **10**, 135-147.
Settleman, J., Narasimhan, V., Foster, L. C. and Weinberg, R. A. (1992). Molecular cloning of cDNAs encoding the GAP-associated protein p190: implications for a signaling pathway from ras to the nucleus. *Cell* **69**, 539-549.
Sgorbissa, A., Benetti, R., Marzinotto, S., Schneider, C. and Brancolini, C. (1999). Caspase-3 and caspase-7 but not caspase-6 cleave Gas2 in vitro: implications for microfilament reorganization during apoptosis. *J. Cell Sci.* **112**, 4475-4482.
Shibusa, T., Shijubo, N. and Abe, S. (1998). Tumour angiogenesis and vascular endothelial growth factor expression in stage I lung adenocarcinoma. *Clin. Cancer Res.* **4**, 1483-1487.
Somasiri, A., Howarth, A., Goswami, D., Dedhar, S. and Roskelley, C. D. (2001). Overexpression of the integrin-linked kinase mesenchymally transforms mammary epithelial cells. *J. Cell Sci*. **114**, 1125-1136.
Songyang, Z., Baltimore, D., Cantley, L. C., Kaplan, D. R. and Franke, T. F. (1997). Interleukin 3-dependent survival by the Akt protein kinase. *Proc. Natl. Acad. Sci. USA* **94**, 11345-11350.
Standaert, M. L., Bandyopadhyay, G., Kanoh, Y., Sajan, M. P. and Farese, R. V. (2001). Insulin and PIP3 activate PKC-zeta by mechanisms that are both dependent and independent of phosphorylation of activation loop (T410) and autophosphorylation (T560) sites. *Biochemistry* **40**, 249-255.
Stratford, S., DeWald, D. B. and Summers, S. A. (2001). Ceramide dissociates 3′-phosphoinositide production from pleckstrin homology domain translocation. *Biochem. J*. **354**, 359-368.
Takahashi, T. and Shibuya, M. (1997). The 230 kDa mature form of KDR/Flk-1 (VEGF receptor-2) activates the PLC-gamma pathway and partially induces mitotic signals in NIH3T3 fibroblasts. *Oncogene* **14**, 2079-2089.
Takahashi, T., Ueno, H. and Shibuya, M. (1999). VEGF activates protein kinase C-dependent, but Ras-independent Raf-MEK-MAP kinase pathway for DNA synthesis in primary endothelial cells. *Oncogene* **18**, 2221-2230.
Terman, B. I., Carrion, M. E., Kovacs, E., Rasmussen, B. A., Eddy, R. L. and Shows, T. B. (1991). Identification of a new endothelial cell growth factor receptor tyrosine kinase. *Oncogene* **6**, 1677-1683.
Tu, Y., Li, F. and Wu, C. (1998). Nck-2, a novel Src homology2/3-containing adaptor protein that interacts with the LIM-only protein PINCH and components of growth factor receptor kinase-signaling pathways. *Mol. Cell. Biol*. **12**, 3367-3382.
Tu, Y., Huang, Y., Zhang, Y., Hua, Y. and Wu, C. (2001). A new focal adhesion protein that interacts with integrin-linked kinase and regulates cell adhesion and spreading. *J. Cell Biol.* **153**, 585-598.
Waltenberger, J., Claesson Welsh, L., Siegbahn, A., Shibuya, M. and Heldin, C. H. (1994). Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *J. Biol. Chem*. **269**, 26988-26995.
Wu, C. (1999). Integrin-linked kinase and PINCH: partners in regulation of cell-extracellular matrix interaction and signal transduction. *J. Cell Sci*. **112**, 4485-4489.
Wu, C., Keivens, V. M., O'Toole, T. E., McDonald, J. A. and Ginsberg, M. H. (1995). Integrin activation and cytoskeletal interaction are essential for the assembly of a fibronectin matrix. *Cell* **83**, 715-724.
Wu, C., Keightley, S. Y., Leung-Hagesteijn, C., Radeva, G., Coppolino, M., Goicoechea, S., McDonald, J. A. and Dedhar, S. (1998). Integrin-linked protein kinase regulates fibronectin matrix assembly, E-cadherin expression, and tumorigenicity. *J. Biol. Chem*. **273**, 528-536.
Wu, L. W., Mayo, L. D., Dunbar, J. D., Kessler, K. M., Baerwald, M. R., Jaffe, E. A., Wang, D., Warren, R. S. and Donner, D. B. (2000). Utilization of distinct signaling pathways by receptors for vascular endothelial cell growth factor and other mitogens in the induction of endothelial cell proliferation. *J. Biol. Chem*. **275**, 5096-5103.
Wymann, M. P. and Pirola, L. (1998). Structure and function of phosphoinositide 3-kinases. *Biochim. Biophys. Acta* **1436**, 127-150.
Xia, P., Aiello, L. P., Ishii, H., Jiang, Z. Y., Park, D. J., Robinson, G. S., Takagi, H., Newsome, W. P., Jirousek, M. R. and King, G. L. (1996). Characterization of vascular endothelial growth factor's effect on the activation of protein kinase C, its isoforms, and endothelial cell growth. *J. Clin. Invest*. **98**, 2018-2026.
Yamaji, S., Suzuki, A., Sugiyama, Y., Koide, Y., Yoshida, M., Kanamori, H., Mohri, H., Ohno, S. and Ishigatsubo, Y. (2001). A novel integrin-linked kinase-binding protein, affixin, is involved in the early stage of cell-substrate interaction. *J. Cell Biol.* **153**, 1251-1264.
Yao, R. and Cooper, G. M. (1996). Growth factor-dependent survival of rodent fibroblasts requires phosphatidylinositol 3-kinase but is independent of pp70S6K activity. *Oncogene* **13**, 343-351.