The Shc-related adaptor protein, Sck, forms a complex with the vascular-endothelial-growth-factor receptor KDR in transfected cells

Andrea J. WARNER*, Jennifer LOPEZ-DEE†, Emma L. KNIGHT*, James R. FERAMISCO† and Sally A. PRIGENT*[1]

*Department of Biochemistry, University of Leicester, Leicester LE1 7RH, U.K., and †UCSD Cancer Center, San Diego, CA 92093, U.S.A.

Despite much progress in recent years, the precise signalling events triggered by the vascular-endothelial-growth-factor (VEGF) receptors, fms-like tyrosine kinase (Flt1) and kinase insert domain-containing receptor (KDR), are incompletely defined. Results obtained when Flt1 and KDR are individually expressed in fibroblasts or porcine aortic endothelial cells have not been entirely consistent with those observed in other endothelial cells expressing both receptors endogenously. It has also been difficult to demonstrate VEGF-induced phosphorylation of Flt1, which has led to speculation that KDR may be the more important receptor for the mitogenic action of VEGF on endothelial cells. In an attempt to identify physiologically important effectors which bind to KDR, we have screened a yeast two-hybrid mouse embryo library with the cytoplasmic domain of KDR. Here we describe the identification of the adaptor protein, Shc-like protein (Sck), as a binding partner for KDR. We demonstrate that this interaction requires phosphorylation of KDR, and identify the binding site for the Src-homology 2 (SH2) domain as tyrosine-1175 of KDR. We have also shown that the SH2 domain of Sck, but not that of Src-homology collagen protein (Shc), can precipitate phosphorylated KDR from VEGF-stimulated porcine aortic endothelial cells expressing KDR, and that an N-terminally truncated Sck protein can associate with KDR, in a phosphorylation-dependent fashion, when co-expressed in human embryonic kidney 293 cells. Furthermore, we demonstrate that in the two-hybrid assay, both Shc and Sck SH2 domains can associate with the related receptor Flt1.

Key words: angiogenesis, receptor tyrosine kinase, signalling, VEGF.

INTRODUCTION

Vascular endothelial growth factor (VEGF) is a key angiogenic growth factor that binds to two receptor tyrosine kinases, fms-like tyrosine kinase (Flt1) and kinase insert domain-containing receptor (KDR) almost exclusively expressed in endothelial cells [1]. The importance of VEGF and its receptors in embryonic vasculogenesis and angiogenesis has been demonstrated by gene knock-out studies in mice, where targeted disruption of either VEGF, or its receptors, leads to embryonic death resulting from vascular defects [2–4]. In the case of the foetal liver kinase (Flk)1-deficient mice, this appears to be due to a failure of blood island formation, whereas in the Flt1-deficient mice, endothelial precursor cells form, but are not organized into vessels. This suggests different roles for Flt1 and KDR in endothelial-cell differentiation and vessel formation. Interestingly, heterozygous embryos lacking only one VEGF allele die *in utero* with cardiovascular defects, indicating that the level of expression of VEGF tightly controls development of the cardiovascular system [4].

In cell-culture models, VEGF is a potent endothelial-cell mitogen, and it induces endothelial cells to form tube-like structures in Matrigel and in collagen gel assays [5,6]. VEGF also stimulates angiogenesis in the rat cornea and chicken chorioallantoic membrane models [7–9]. Since angiogenesis plays an important role in the growth and metastasis of tumours, much attention has focused on the development of inhibitors of this process for tumour therapy [10]. Inhibition either of VEGF activity by neutralizing antibodies, or of KDR through a dominant negative approach, has already shown promise as a therapeutic strategy for inhibition of tumour growth in animals [11–13]. Moreover, clinical trials are already underway using agents which are relatively selective for inhibition of VEGF receptors as a potential therapy for cancer [14].

Despite these advances, the downstream effector signalling pathways utilized by VEGF receptors remain poorly defined. Both Flt1 and KDR autophosphorylate *in vitro*; however, phosphorylation of Flt1 in response to VEGF stimulation of intact endothelial cells has been difficult to demonstrate. VEGF treatment of porcine aortic endothelial (PAE) cells transfected with KDR promotes an increase in mitogen-activated protein (MAP) kinase activation, DNA synthesis, changes in cell morphology and membrane ruffling, whereas stimulation of Flt1-expressing PAE cells induces no such responses [15,16]. Such observations have led to the suggestion that KDR may be the more important receptor for mediating the mitogenic effects of VEGF. VEGF-induced activation of Flt1 has, however, been reported to be involved in the migration of monocytes [17,18], and MAP kinase activation by Flt1 has been reported in response to the VEGF-related growth factor, placental growth factor (PlGF) [16]. Various components of receptor tyrosine kinase signalling pathways have been shown to be involved in VEGF signalling. However, experiments performed in a variety of endothelial cell systems [bovine aortic endothelial cells, human umbilical-vein endothelial cells (HUVECs), liver sinusoidal en-

Abbreviations used: Shc, Src-homology collagen protein; Sck, Shc-like protein; VEGF, vascular endothelial growth factor; KDR kinase insert domain-containing receptor (human VEGF receptor 2); Flk1, foetal liver kinase 1 (mouse VEGF receptor 2); Flt1, fms-like tyrosine kinase (VEGF receptor 1); SH2, Src-homology 2; PTB, phosphotyrosine-binding; MAP kinase, mitogen-activated protein kinase; PI-3K, phosphoinositide 3-kinase; PLCγ, phospholipase Cγ; SHP, Src-homology 2 phosphatase; GST, glutathione S-transferase; PAE cells, porcine aortic endothelial cells; PlGF, placental growth factor; JNK, c-Jun N-terminal kinase; PKC, protein kinase C; HEK-293 cells, human embryonic kidney 293 cells; HUVECs, human umbilical-vein endothelial cells; HA, haemagglutinin; EGF, epidermal growth factor.

[1] To whom correspondence should be addressed (e-mail sap8@le.ac.uk).

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dothelial cells, human microvascular endothelial cells] have produced differing results [19–21].

Studies using inhibitors of phosphoinositide 3-kinase (PI-3K) have provided evidence for its involvement in VEGF-induced survival of endothelial cells [22]. A direct increase in PI-3K activity in response to VEGF has been demonstrated by some investigators [23], while others have reported an absence of activation in response to VEGF [24]. Phospholipase Cγ (PLC)γ has been reported to be phosphorylated in response to VEGF [25], and to associate with both Flt1 and KDR in a yeast two-hybrid assay [26]. The protein tyrosine phosphatases Src-homology 2 phosphatase (SHP)-1 and -2 have been demonstrated to associate with KDR in a VEGF-dependent fashion in transfected PAE/KDR cells [27], whereas Yes and Fyn have been reported to selectively bind Flt1, albeit rather weakly [15]. Other signalling components, such as Grb2, Src-homology collagen protein (Shc), Crk and Nck, have been reported to bind one or both of the receptors in certain assays [28,29].

In addition to the evidence supporting the involvement of known effectors in VEGF signalling there is evidence to suggest that effectors may be expressed in endothelial cells that are absent in other cell types such as fibroblasts. VEGF-stimulated NIH3T3 fibroblasts over-expressing KDR were very weakly mitogenic compared with the response in rat sinusoidal endothelial cells [25]. Activation of the MAP kinase pathway in these cells occurred significantly more slowly than in sinusoidal endothelial cells, and occurred as a consequence of PLCγ and protein kinase C (PKC) activation. This suggests that endothelial-cell-specific proteins couple KDR to the Ras–MAP kinase pathway. Further support for the existence of endothelial cell-specific effectors comes from studies using immobilized peptides, encompassing autophosphorylation sites on Flt1, to purify proteins from a variety of cell types [29]. A novel 27 kDa Grb2/Grap-like molecule, and unidentified 74 and 75 kDa proteins were purified in this way, and reported to be selectively expressed in endothelial cells [29].

In order to identify effectors which bind to KDR, we have begun screening a mouse embryo cell-derived cDNA library, to identify VEGF receptor-interacting proteins using the yeast two-hybrid system. Here we describe the association of the Src-homology 2 (SH2) domain of the Shc-like protein (Sck)/ShcB adaptor protein with the cytoplasmic domain of KDR that occurs in a phosphorylation-dependent manner. KDR did not bind to the corresponding region of ShcA; therefore KDR may utilize ShcB, but not ShcA, to transduce its mitogenic response.

EXPERIMENTAL

Generation of two-hybrid constructs

RNA was extracted from human dermal microvascular endothelial cells (TCS, Botolph Claydon, Buckingham, U.K.) using the RNAeasy kit from Qiagen. mRNA and cDNA were made by the First-strand cDNA Synthesis Kit (Pharmacia) using random primers, and the cDNA encoding the cytoplasmic domain of KDR was amplified by PCR using *Taq* extender (Promega), and the following primers (5′-TCATGGATCCATGAACTCCCATTGGATGAACAT-3′; 5′-TGGATGGATCCTTTTAAACAGGAGGAGAG-3′). The 1.6 kb fragment obtained was subcloned into the *Bam*H1 site of the vector pBTM116 [30] to generate a fusion with the LexA DNA-binding domain. cDNA encoding the cytoplasmic domain of Flt1 was amplified by PCR from the full-length human cDNA, kindly provided by Dr L. Claesson-Welsh (Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden), using the following primers (5′-CAGATGAATTCCCTTTGGATGAGCAGTGTGAG-3′; 5′-GCTTCGGATCCAACTCTAGATGGGTGGGGTGGA-3′), and was cloned into the *Bam*H1 and *Sal*1 sites of pBTM116. cDNA encoding the SH2 domain of Shc was amplified from the full-length cDNA (provided by Dr E. Skolnik, Department of Pharmacology, Skirball Institute for Medical Biochemistry and Microbiology, New York, NY, U.S.A.) using the following primers (5′-CCATGTGGATCCAGCTCCGAGGGGAGCCC-3′; 5′-CTAGGGGAATTCACAGTTTCCGCTCCAC-3′) and cloned into the *Bam*H1/*Eco*R1 sites of pVP16.

Antibodies

Mouse monoclonal antibodies recognizing phosphotyrosine (PY-20, Rc-20, and PY-20 conjugated to agarose) were obtained from Transduction Laboratories (San Diego, CA, U.S.A.). The rabbit polyclonal (sc-504) and monoclonal (sc-6251) antibodies recognizing KDR were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), as were the goat anti-LexA antibody (sc-1725), the mouse anti-phosphotyrosine antibody (PY99), the mouse anti-(glutathione S-transferase) (anti-GST) antibody (sc-138), the rabbit anti-His antibody (sc-803), and all of the horseradish peroxidase-conjugated antibodies. The rat anti-haemagglutinin (anti-HA) antibody was from Roche (clone 3F10), and the rabbit anti-Shc antibody was from Upstate Biotechnology.

Yeast culture, transformation and two-hybrid screen

The L40 yeast strain (*MATa trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-LacZ*) [30] was routinely propagated on YEPD plates, and transformed according to the lithium acetate/poly(ethylene glycol) method described previously [31]. Detailed protocols for media preparation, two-hybrid screening and subsequent isolation of plasmids from positive clones have been described by Bartel et al. [32]. Briefly, the pBTM116 construct encoding the cytoplasmic domain of KDR was used to screen a 8.5–9.5-day mouse embryo cDNA library constructed by Dr S. Hollenberg (Fred Hutchinson Cancer Center, Seattle, WA, U.S.A.) [33]. L40 transformants were plated on to selective plates lacking leucine and tryptophan to select for the bait and library plasmids, lacking histidine to select for expression of the HIS3 reporter gene, and containing 3 mM 3-aminotriazole to suppress a low level of constitutive activation of the HIS3 reporter by the bait plasmid. Yeast colonies growing after 10 days were tested for their ability to activate the β-galactosidase reporter by filter assays, and library plasmids were isolated from 'positive' yeast as described [32]. Isolated library plasmids were retransformed into yeast together with lamin/pBTM116 to test for non-specific activation of the reporter genes. Inserts were sequenced using the Sequenase 2.0 DNA sequencing kit (Amersham) and the primer 5′-GAGTTTGAGCAGATGTTTA-3′.

Preparation of yeast extracts for immunoprecipitation or Western blotting

L40 yeast transformed with the KDR construct, or pBTM116, were plated on to selection plates lacking tryptophan, and colonies were picked and grown overnight in liquid selection medium. For preparation of extracts to be used for immunoprecipitation, 10 ml of culture was pelleted by centrifugation for 5 min at 700 ***g***. Yeast were resuspended in 300 μl of ice-cold TBS (50 mM Tris/150 mM NaCl, pH 7.4) containing protease and

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Table 1 Mutagenesis primers

Mutation	Forward primer	Reverse primer
KDR		
Y951F	5′CCGTCAAGGGAAAGAATTCGTTGGAGCAATCC3′	5′GGATTGCTCCAACGAATTCTTTCCCTTGACGG3′
Y996F	5′GAAGCTCCTGAAGATGAATTCAAGGACTTCCTGACC3′	5′GGTCAGGAAGTCCTTGAATTCATCTTCAGGAGCTTC3′
Y1054F	5′GCTTGGCCCGGGATGAATTCAAAGATCCAGATTATG3′	5′CATAATCTGGATCTTTGAATTCATCCCGGGCCAAGC3′
Y1059F	5′GGATATTTATAAAGATCCAAGCTTTGTCAGAAAAGGAG3′	5′CTCCTTTTCTGACAAAGCTTGGATCTTTATAAATATCC3′
Δ1	5′GCTCAGCAGGATGGCTAAGCTTACATTGTTCTTCCG3′	5′CGGAAGAACAATGTAAGCTTAGCCATCCTGCTGAGC3′
Δ2	5′CCCCAAATTCCATTAAGCTTACACAGCAGGAATCAG3′	5′CTGATTCCTGCTGTGTAAGCTTAATGGAATTTGGGG3′
Y1175F	5′GCAGGATGGCAAAGAATTCATTGTTCTTCCG3′	5′CGGAAGAACAATGAATTCTTTGCCATCCTG3′
kin—	5′GCAGGACAGTAGCAGTCGACATGTTGAAAGAAGG3′	5′CCTTCTTTCAACATGTCGACTGCTACTGTCCTGC3′
FLT1		
kin—	5′GGACTGTGGCTGTGGCCATGCTGAAAGAGGG3′	5′CCCTCTTTCAGCATGGCCACAGCCACAGTCC3′

phosphatase inhibitors. An equal volume of acid-washed glass beads (425–600 μm diameter) was added and yeast were sonicated ten times for 10 s on ice. The lysate was vortex-mixed vigorously, the supernatant removed and the beads washed in an equal volume of TBS/0.1% Triton X-100 (TBST), which was pooled with the initial supernatant. Tyrosine-phosphorylated proteins were precipitated overnight at 4 °C with PY20 conjugated to agarose (Transduction Laboratories).

Where total yeast extracts were to be analysed directly using SDS/PAGE, 2.5 ml of liquid culture was pelleted and resuspended in 1 ml of 0.25 M NaOH/1% β-mercaptoethanol. Following a 10 min incubation on ice, 0.16 ml of 50% trichloroacetic acid was added and the extracts were incubated for a further 10 min followed by a 10 min centrifugation at 10000 ***g***. Pellets were resuspended in 1 ml of ice-cold acetone, vortex-mixed vigorously, and subjected to centrifugation as above. Recovered pellets were air-dried and resuspended in 200 μl of SDS sample buffer for analysis on SDS/PAGE.

Site-directed mutagenesis of KDR and Flt1

Point mutations were introduced at known autophosphorylations sites of KDR (tyrosine-954, tyrosine-996, tyrosine-1054 and tyrosine-1059) [34], and the predicted ATP-binding sites of KDR and Flt1. Mutagenesis was performed using the QuikChange kit supplied by Stratagene. In addition, two deletion mutants were constructed by incorporating stop codons into the KDR sequence using the QuikChange protocol to produce proteins lacking portions of the C-terminal tail. The sequences of the oligonucleotides used for the mutagenesis are shown in Table 1. A further point mutation at tyrosine-1175 of KDR was introduced later. Sequences of the mutants were verified by DNA sequencing.

Expression of Sck and Shc SH2 domains as GST-fusion proteins

The Sck SH2 domain was excised from pVP16 with *Bam*H1 and *Eco*R1, and subcloned into pGEX-3X (Pharmacia) for expression as a GST-fusion protein. The Shc-SH2 domain was described previously [35]. Both fusion proteins were expressed in DH5α by induction of expression with 0.2 mM isopropyl β-D-thiogalactoside. Immobilization on glutathione–agarose beads was performed according to the protocol outlined by Pharmacia. Following extensive washing, the GST-fusion proteins were left bound to the beads for precipitation experiments. The amount of protein immobilized on the beads was estimated by analysis of a defined volume by SDS/PAGE and Coomassie Brilliant Blue staining, alongside defined amounts of BSA standard.

Phosphopeptide synthesis and phosphatase treatment

A peptide (PY Pep1175) containing the sequence surrounding phosphorylated tyrosine-1175 of KDR (RQIKIWFQNRRMKWKKQDGKDpYIVLPIS) was synthesized by solid-phase Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry, using an Applied Biosystems 431A instrument. The N-terminal 16 amino acids correspond to a sequence from the Antennapedia homeodomain protein. A control phosphopeptide (PY Pep951) corresponding to the sequence surrounding tyrosine-951 (RQGKDpYVGAIP) was used for comparison in the initial competition experiments. For dephosphorylation, 150 μg of PY Pep1175 was incubated at 30 °C, for 24 h, with 6000 units of Lambda protein phosphatase in 200 μl of the supplied buffer (New England Biolabs, Hitchin, Hertfordshire, U.K.). Similarly, peptide was incubated in the absence of enzyme. An aliquot of dephosphorylated and phosphorylated peptide was dot-blotted on to nitrocellulose and cross-linked with 0.05% glutaraldehyde in PBS/Tween (PBST) for 30 min. The nitrocellulose was rinsed four times with PBST, blocked overnight in 5% fat-free milk/TBST, and probed with anti-phosphotyrosine antibody PY20 (Transduction Labs; 1:1000) and mouse horseradish peroxidase-conjugated secondary antibody. The ECL® chemiluminescent substrate system (Amersham) was used to visualize bound antibody.

Cell culture

Human embryonic kidney (HEK)-293 cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% (v/v) fetal-calf serum and 10 mg/ml streptomycin and 100 units/ml penicillin. PAE cells, either untransfected or stably expressing KDR, were provided by Dr L. Claesson-Welsh [15], and were routinely cultured in Ham's nutrient mix F12 medium containing 10% (v/v) fetal-calf serum, non-essential amino acids and sodium pyruvate (supplied as a 100 × stock solution from Gibco BRL), and antibiotics as above.

Generation of a His-tagged Sck clone for transfection into HEK cells

An IMAGE clone (979979) [36] was identified which contained a sequence corresponding to the 3′ untranslated region of human Sck. This clone was obtained from the MRC Human Genome Mapping Resource Centre, and the 5′ sequence confirmed by DNA sequencing. The 2056 bp insert was subcloned into the *Eco*R1/*Xho*1 sites of pcDNA3.1 HisC for expression in HEK-293 cells as a His-tagged fusion protein.

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HEK-293 transfections

The day before transfection, HEK-293 cells were seeded at a density of 2×10^6 cells/9 cm-diameter Petri dish. The following day cells were transfected in the presence of serum with constructs encoding HA-tagged mouse Flk1/KDR or HA-tagged human Flt1 [37], either alone or together with Sck-EST-pcDNA3.1 HisC (described above), using fugene reagent (Roche), according to the manufacturer's protocol. At 24 h after transfection the medium was replaced with serum-free medium, and the cells were used for experiments the next day.

VEGF stimulation

Prior to VEGF treatment, cells were incubated in the appropriate growth medium without fetal calf serum for 24 h. Cells were incubated for 5 min in PBS containing 100 μM Na_3VO_4 and were then stimulated with 50–100 ng/ml $VEGF_{165}$ (R & D systems, Abingdon, Oxfordshire, U.K.) and 100 μM orthovanadate for 1 h at 4 °C, followed by 8 min at 37 °C, as described previously [15]. For comparison, cells were treated similarly with orthovanadate in the absence of VEGF.

Precipitations of KDR with antibodies and GST-fusion proteins

For experiments using PAE and PAE/KDR cells, the lysate from one 15 cm dish was divided into three tubes for precipitation with GST-Sck-SH2, GST-Shc-SH2 or anti-KDR (sc-504). Following VEGF treatment, cells were lysed in 1 % Triton X-100/50 mM Tris/HCl (pH 7.4)/150 mM NaCl/5 mM EGTA/25 mM benzamidine/100 μM Na_3VO_4, 100 mM NaF/1 mM PMSF/10 μg/ml each aprotinin + leupeptin + pepstatin. Cell lysates were centrifuged at 25000 ***g***, at 4 °C, for 5 min for clarification. Proteins were precipitated with 5 μg of GST–Sck-SH2, or GST–Shc-SH2 bound to glutathione–agarose, or 5 μg of anti-KDR polyclonal antibody bound to Protein A–agarose (sc-504; Santa Cruz) for comparison. For experiments performed on transfected HEK-293 cells, 2 μg of rabbit anti-His antibody bound to protein A–agarose (2.5 mg/precipitation), or 5 μg anti-HA bound to Protein G–agarose was used per precipitation. Protein A– or G–antibody complexes were pre-formed by incubation together, at room temperature for 1 h, and washed with 1 ml of PBS, prior to incubation with cell lysate overnight, at 4 °C.

Immunoprecipitates were washed four times with TBS/0.1 % Triton X-100, and pellets boiled for 5 min in SDS-containing sample buffer for analysis by SDS/PAGE [38]. Proteins were transferred to nitrocellulose for probing with appropriate antibodies, and immunoblots were developed using the ECL® chemiluminescent substrate detection system (Amersham).

For peptide-competition studies, GST–Sck-SH2 domain bound to glutathione–Sepharose was pre-incubated with increasing concentrations of either phosphorylated or dephosphorylated PY Pep1175 (0, 1, 10 and 100 μg), or phosphorylated PY Pep951 (0.4, 4 and 40 μg) for 1 h prior to addition of PAE/KDR cell lysate, prepared as described above.

RESULTS

Phosphorylated KDR interacts with Sck-SH2 domain, but not Shc-SH2 domain, in yeast, whereas Flt1 interacts with both Shc and Sck SH2 domains

Since signalling cascades are generally initiated by phosphotyrosine-dependent interactions, we first verified that the KDR receptor was phosphorylated in yeast. A band of approx. 116 kDa corresponding to the KDR–LexA fusion protein was clearly visible when anti-phosphotyrosine immunoprecipitates were analysed by SDS/PAGE and Western blotting with an antibody recognizing KDR (Figure 1).

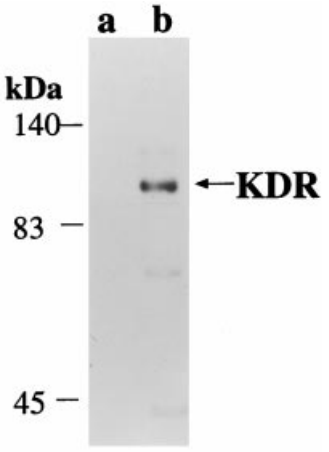

Figure 1 The KDR cytoplasmic domain is phosphorylated when expressed in yeast

Extracts were prepared from yeast transformed with a control vector (lane a) or yeast expressing KDR (lane b). Proteins were immunoprecipitated with an anti-phosphotyrosine antibody and immunoblots were probed with an antibody recognizing KDR.

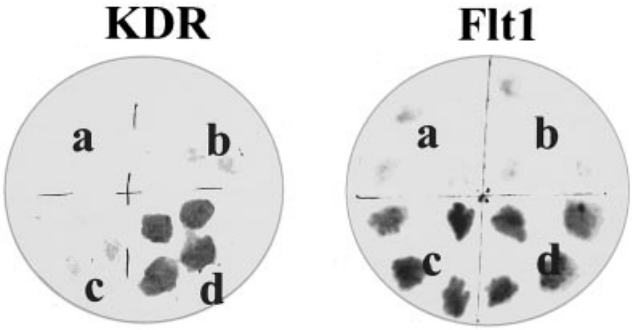

Figure 2 Interaction of KDR and Flt1 with Sck and Shc-SH2 domains

Yeast was transformed with LexA-Fusions of wild-type, or kinase-inactive KDR or Flt1 together with the Sck, or Shc-SH2 domains fused to the pVP16 DNA binding domain as follows: (a) kinase-inactive receptor + Shc-SH2; (b) kinase-inactive receptor + Sck-SH2; (c) wild-type receptor + Shc-SH2; (d) wild-type receptor + Sck-SH2. Transformed yeasts were tested for their ability to activate the LacZ reporter by β-galactosidase filter assays.

After screening 100000 clones with this construct, a single library plasmid was isolated which interacted with KDR, thereby activating both the HIS3 and β-galactosidase reporters. DNA sequencing revealed that this cDNA corresponded to the SH2 domain of Sck/ShcB (corresponding to residues 440–540 of the human sequence; GenBank® accession no. AB00145 [39]). Since Sck is closely related to the adaptor protein Shc, we used PCR to generate a construct encoding the exact corresponding region of Shc and tested this for interaction with KDR, and also with Flt1 (Figure 2). Only the Sck-SH2 domain interacted with KDR, whereas both Sck and Shc SH2 domains interacted with Flt1 (Figure 2). In order to establish the phosphorylation-dependence of the association of Sck and Shc with KDR and Flt1, point mutants of KDR and Flt1 were constructed and expressed, in which the predicted critical lysine residues at the ATP-binding

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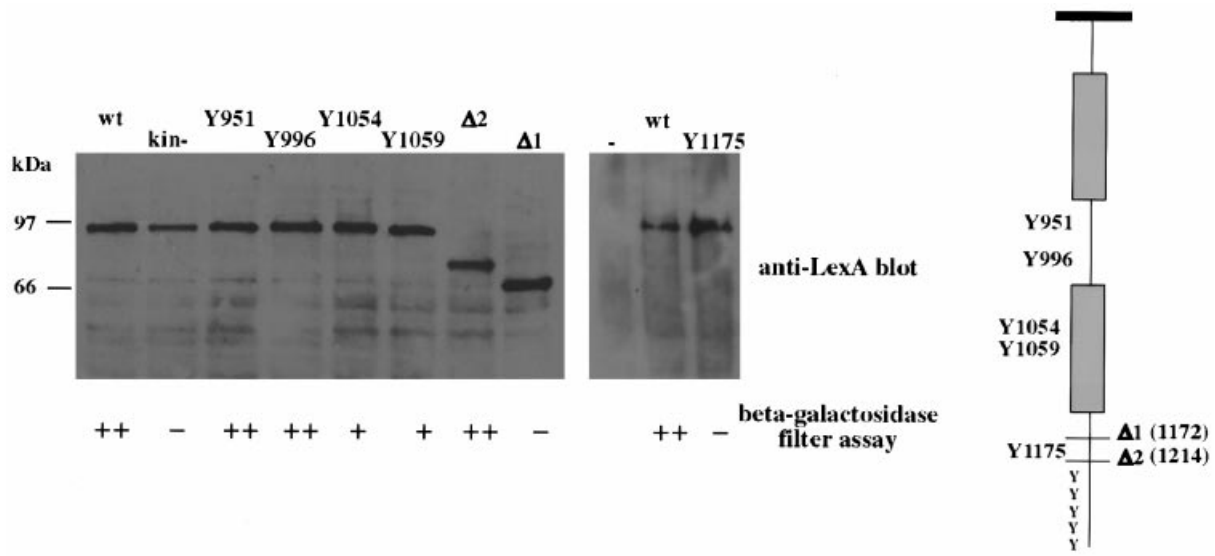

Figure 3 Identification of Sck-binding site on KDR by mutational analysis

The Sck-SH2 domain was tested for its ability to interact with wild-type KDR (wt), kinase inactive KDR (kin−), point mutants in which tyrosine residues corresponding to previously reported autophosphorylation sites were changed to phenylalanine (tyrosine-951, tyrosine-996, tyrosine-1054, tyrosine-1059 and tyrosine-1175), and deletion mutants lacking portions of the C-terminal tail (Δ1 and Δ2). To control for equivalent expression of mutants, immunoblots were performed on extracts of transformed yeast with an anti-LexA antibody.

sites (lysine-868 and lysine-861, respectively) were changed to aspartic acid. These kinase-inactive mutants failed to interact with Sck or Shc (Figure 2).

Sck-SH2 binds to tyrosine-1175 on KDR

To identify the binding site for Sck on KDR, point mutants were made in which tyrosine residues previously reported to be phosphorylated (tyrosine-951, tyrosine-996, tyrosine-1054 and tyrosine-1059) were changed to phenylalanine. In addition, two deletions were constructed by introducing stop codons at positions corresponding to residues 1172 (Δ1) and 1214 (Δ2) of the human KDR sequence. Expression of the mutants in yeast was confirmed by immunoblotting with an anti-LexA antibody (Figure 3). All of these mutants interacted with the Sck-SH2 domain, except Δ1. The tyrosine-1054 and tyrosine-1059 mutants showed reduced binding, presumably because phosphorylation of these sites contributes to the catalytic activity of KDR [40,41]. A single tyrosine residue was present between residues 1172 and 1214 at position tyrosine-1175. In order to confirm that this was the site of association of Sck, a further point mutant at tyrosine-1175 was constructed and expressed, which failed to interact with KDR (Figure 3).

Sck-SH2 is able to precipitate phosphorylated KDR from mammalian cells

Since KDR appeared to selectively bind to the Sck-SH2 domain, we sought to verify that this domain was also able to selectively precipitate KDR expressed in mammalian cells. While the Sck-SH2 domain precipitated phosphorylated KDR from transfected PAE cells which had been treated with VEGF, the Shc-SH2 domain did not (Figure 4a). To assess the amount of GST-fusion protein used in each precipitation, blots were stripped and reprobed with an antibody recognizing GST. More fusion protein was actually used for precipitations with GST–Shc compared with GST–Sck, thus the inability to precipitate KDR with Shc-SH2 domain was not simply due to insufficient immobilized Shc-SH2 domain being present (Figure 4a). The GST–Shc was able to precipitate epidermal growth factor (EGF) receptor from transfected NIH 3T3 cells, and therefore did contain a functional SH2 domain [35]. Experiments were not performed with PAE cells expressing Flt1, as we are unable to detect phosphorylation of Flt1 in these cells. In order to verify that tyrosine-1175 was a binding site for Sck, a phosphotyrosine-containing peptide, PY Pep1175, was synthesized encompassing resides 1170–1181 of KDR, and its ability to compete with KDR for binding to GST–Sck was tested. GST–Sck was unable to precipitate KDR in the presence of 10 μg of PY Pep1175 (Figure 4b). Pretreatment of the peptide with Lambda phosphatase removed approx. 90% of the phosphate as determined by dot-blots using anti-phosphotyrosine antibodies (Figure 4c). The dephosphorylated peptide was no longer able to inhibit KDR binding to Sck (Figure 4b). To confirm that the inhibitory effect of PY Pep1175 peptide on KDR binding to Sck was dependent on the sequence surrounding the phosphotyrosine residue, experiments were repeated using a control phosphopeptide incorporating the sequence surrounding tyrosine-951 (Figure 4d). This peptide showed no inhibitory effect on KDR binding to Sck.

Shc is found in complexes with KDR, but not Flt1, in transfected HEK-293 cells

Since the two-hybrid results indicated that Shc, as well as Sck, could interact with active Flt1, we examined whether Shc could associate with Flt1 in intact cells. To maximize the sensitivity of our experiments we used HA-tagged constructs of Flk1/KDR and Flt1 expressed in HEK-293 cells, to facilitate detection of both receptors with an anti-HA antibody. Initially we aimed to determine whether or not we could induce receptor phosphorylation following ligand stimulation, and whether or not endogenous Shc co-immunoprecipitated with either receptor. While both receptors could be expressed at high levels in HEK-293 cells, only KDR exhibited an increase in tyrosine

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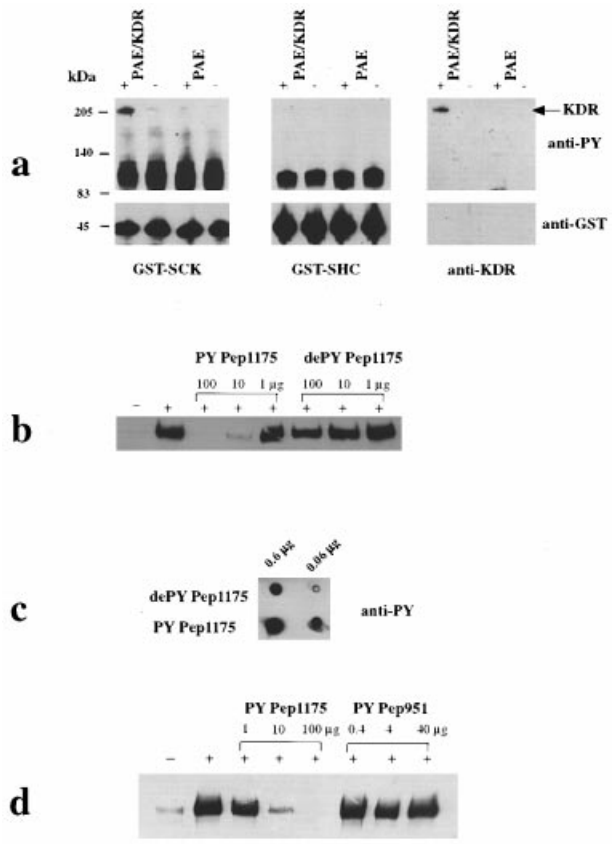

Figure 4 GST–Sck-SH2, but not GST–Shc-SH2, precipitates phosphorylated KDR: binding is inhibited by a synthetic phosphopeptide encompassing tyrosine-1175

(**a**) The Sck-SH2 domain was expressed as a GST-fusion protein in *Escherichia coli*. A 5 μg portion of fusion protein was used to precipitate proteins from PAE cells transfected with KDR (PAE/KDR), which had either been treated with VEGF prior to lysis (+), or were untreated (−). For comparison, precipitations were performed using a GST–Shc-SH2 domain fusion protein and an anti-KDR polyclonal antibody, and were performed in parallel with extracts of parental PAE cells. Precipitating proteins were analysed by SDS/PAGE and immunoblotting with an anti-phosphotyrosine antibody (upper panels). Blots were stripped and re-probed with an anti-GST antibody to control for the amount of fusion protein used for the precipitations (lower panels). (**b**) KDR was precipitated with GST–Sck from VEGF-treated (+) or untreated (−) PAE/KDR cells in the presence of increasing amounts of tyrosine-phosphorylated peptide PY-Pep1175. For comparison, the peptide was treated with Lambda phosphatase before use (dePY-Pep1175). The presence of KDR was detected using an anti-KDR monoclonal antibody. (**c**) $>$ 90% dephosphorylation of PY-Pep1175 was demonstrated by dot-blots using an anti-phosphotyrosine antibody. (**d**) To test the specificity of the inhibitory effect of PY-Pep1175 on KDR binding to Sck, precipitations were repeated, as described above, in the presence of increasing amounts of an unrelated tyrosine phosphopeptide PY-Pep951.

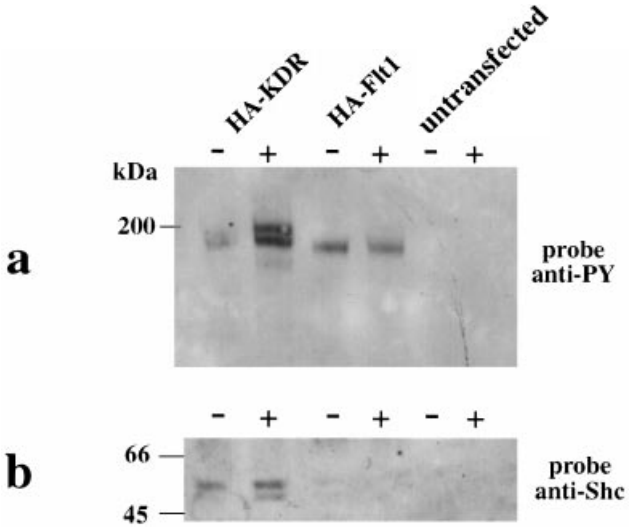

Figure 5 VEGF-induced phosphorylation of KDR, but not Flt1, when expressed in HEK-293 cells

Proteins were precipitated from HEK-293 cells, either untransfected, or transfected with HA-KDR or HA-Flt1 using an anti-HA antibody. Cells were either stimulated (+) or not stimulated (−) with VEGF prior to lysis. Precipitated proteins were analysed by SDS/PAGE and immunoblotting with an anti-phosphotyrosine antibody (**a**). To identify the presence of Shc in complexes with the receptors, immunoblots were stripped and probed with an anti-Shc antibody (**b**).

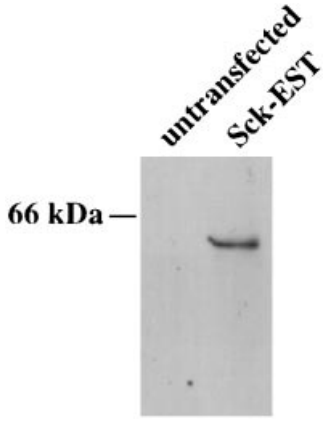

Figure 6 Expression of a His-tagged truncated Sck protein in HEK-293 cells

HEK-293 cells were transfected with an N-terminally truncated Sck protein encoding a polyhistidine epitope tag. Whole-cell lysates were probed with an anti-His antibody.

phosphorylation in response to VEGF (Figure 5). Immunoprecipitates of receptors from VEGF-stimulated HEK-293 cells, probed with an antibody recognizing Shc, demonstrated that a very small amount of Shc was found in complexes containing KDR, but there was little increase in the amount of Shc recruited to these complexes in response to receptor activation. In these experiments we could not detect Shc in Flt1-containing complexes, despite a low level of Flt-1 tyrosine phosphorylation. However, since we were unable to induce phosphorylation by VEGF stimulation, we cannot draw any conclusions as to whether Shc is able to associate with Flt1 when it becomes activated.

Activated KDR binds Sck when expressed in HEK-293 cells

Having demonstrated that a fusion protein corresponding to the Sck-SH2 domain was able to precipitate phosphorylated KDR from cell lysates, we wished to confirm that this interaction could occur *in vivo*. To facilitate the detection of Sck when expressed in cells, we generated an epitope-tagged Sck protein (Sck-EST) from an EST (expressed sequence tag) clone which encoded amino acids 149–540 (C-terminus) of the published human partial Sck sequence (GenBank® accession no. AB00145 [39]). This protein encoded a polyhistidine sequence (His_6) and the Xpress epitope tag at the N-terminus and produced a protein of approx.

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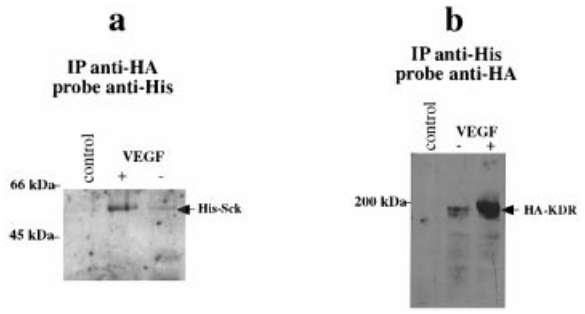

Figure 7 Co-immunoprecipitation of KDR and Sck from transfected HEK 293 cells

HEK-293 cells co-transfected with HA-KDR and His-Sck-EST were treated with VEGF prior to lysis (+) or were untreated (−). A third plate of untransfected cells was treated with VEGF. KDR and Sck proteins were immunoprecipitated with anti-HA (**a**) and anti-His antibodies (**b**) respectively, and associating Sck (**a**) and KDR (**b**) were detected on immunoblots with appropriate antibodies as indicated.

55 kDa when expressed in HEK-293 cells, which could be detected on immunoblots of whole cell lysates with antibodies recognizing either epitope tag (Figure 6).

Sck-EST was immunoprecipitated from HEK-293 cells which had been transiently co-transfected with HA-tagged KDR and His-tagged Sck-EST (Figure 7b). In the absence of VEGF treatment a faint doublet of approx. 205 kDa, corresponding to KDR, was apparent in the HA-KDR transfected cells, which was absent in immunoprecipitates from untransfected cells. Following incubation with 50 ng/ml VEGF there was an increase in precipitation of both molecular-mass species with an antibody recognizing the co-expressed Sck fusion protein. Equal expression of HA-KDR and Sck-EST in both stimulated and unstimulated extracts was confirmed by SDS/PAGE and Western-blot analysis of whole cell lysates (results not shown). Experiments were also performed using an HA-tagged protein which was unrelated to KDR, HA-JNK (where JNK is c-Jun N-terminal kinase). Despite very high levels of expression of this protein together with His-Sck-EST, no HA-JNK could be detected in Sck immunoprecipitates, indicating that the binding was not simply an artefact of overexpression (results not shown). To confirm the association between Sck and KDR, KDR was immunoprecipitated from transfected cells and treated with VEGF as above. The presence of Sck was detected using the anti-His antibody (Figure 7a). While a small amount of Sck associated with KDR in the unstimulated state, an increase was observed upon VEGF treatment.

DISCUSSION

Studies performed on a variety of endothelial and non-endothelial cells have led to the suggestion that KDR may be the receptor primarily responsible for the mitogenic effects of VEGF in endothelial cells, and that effectors other than those commonly found in fibroblasts (e.g. Grb2, Shc, PI-3K) may be involved in coupling KDR to the MAP kinase cascade. We sought to identify such effectors using a yeast two-hybrid approach to screen for proteins interacting with KDR. Since the function of KDR is particularly important during vascular development we used a cDNA library derived from 8.5–9.5-day mouse embryos to identify binding partners for KDR. After screening 100000 clones, only one cDNA was identified which interacted strongly with the KDR cytoplasmic domain, this corresponding to the SH2 domain of the Shc-related protein, Sck. Sck was first identified as an EST clone with sequence similarity to the adaptor Shc [42], and possible full-length, or almost full-length, cDNAs were subsequently identified by screening brain cDNA libraries or genomic libraries for homologues of Shc [39,43,44]. It is noteworthy, however, that the N-terminal sequence of Sck was not reported by two groups [39,43], and the third group reported a sequence identified by rapid-amplification-of-cDNA-ends ('RACE') PCR which is inconsistent with the other reports [44]. While Shc is a ubiquitously expressed adaptor protein which couples many receptor tyrosine kinases to the Ras–MAP kinase pathway [45], Sck has been reported to be expressed at lower levels in most tissues, but is particularly abundant in heart, liver, pancreas and prostate [39]. Here we have shown that, in yeast, the Sck SH2 domain bound to tyrosine-1175 of KDR, contained within the motif YIVLP, but the related Shc-SH2 domain did not. It will be important to confirm that tyrosine-1175 represents a VEGF-induced phosphorylation site in mammalian cells. Our observation that GST–Sck was able to precipitate full-length KDR from VEGF-stimulated mammalian cells demonstrates that the interaction site is phosphorylated under these conditions. Furthermore, the ability of a phosphopeptide corresponding to the sequence surrounding tyrosine-1175, but not an unrelated phosphopeptide, to compete for this binding suggests that in intact cells, the interaction site is indeed tyrosine-1175. Evidence from other groups suggests that, in addition to the four auto-phosphorylation sites identified by expression of the KDR cytoplasmic domain in bacteria (tyrosine-951, tyrosine-996, tyrosine-1054 and tyrosine-1059), additional sites exist *in vivo* which are important for VEGF-induced phosphorylation of PLCγ, focal adhesion kinase and paxillin [40]. It is tempting to speculate that tyrosine-1175 may be one of these functionally important phosphorylation sites.

Two different groups have attempted to identify a consensus binding sequence for Sck-SH2 domain binding, with limited success. However, a hydrophobic residue seems to be essential in the +3 position relative to the phosphotyrosine [43,44], and in one of these studies, leucine was the preferred residue in this position, being present in nine out of 24 peptide sequences analysed. Interestingly, two of the 24 peptides were very similar to the binding motif on KDR, and contained the sequences YDVLP and YSVIP. Our inability to detect direct binding of the Shc-SH2 to this site on KDR suggests that there are motifs which can be selectively bound by distinct Shc family members, and in that sense they are not functionally redundant. Despite our inability to detect direct association of Shc-SH2 domain with KDR in the two-hybrid assay, when KDR is overexpressed in HEK-293 cells it is able to bind a very small amount of Shc. This binding is only slightly stimulated by VEGF, and is difficult to detect. Constructs encoding the phosphotyrosine-binding (PTB) domain of Shc were also unable to associate with KDR in our two-hybrid assay (results not shown), which is not surprising, since KDR lacks the well-characterized binding motif for the Shc-PTB domain (NPXY). It is possible, therefore, that Shc may indirectly associate via a secondary adaptor. The significance of this weak association is unclear at present. Kroll and Waltenberger [27] reported Shc binding to KDR using sensitive *in vitro* kinase assays to detect the presence of KDR in Shc immunoprecipitates, while others have suggested that Shc has little or no role in VEGF-stimulated endothelial-cell signalling [21]. Interestingly, Thakker and co-workers recently reported that KDR associates constitutively with the p85 subunit of PI-3K [23]. KDR lacks the characteristic YXXM motif which, when phosphorylated, is the preferred binding site for the p85 SH2 domain. It is possible that both Shc and PI-3K are part of a

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larger complex constitutively associated with KDR, the components of which become activated by phosphorylation upon VEGF binding to KDR. In contrast with the situation with Shc, we were able to detect a clear ligand-induced association of an N-terminally truncated Sck protein when this was co-expressed with KDR. This protein lacked a portion of the PTB domain, rendering it non-functional, but possessed an intact collagen-homology domain 1 and SH2 domain. It will be important to determine the downstream events triggered by this association, and we are currently trying to isolate a full-length Sck cDNA to facilitate these studies.

We have not identified the binding site for Shc or Sck on Flt1. However, the YIVLP motif is not present in Flt1, and the ability of both Shc and Sck-SH2 domains to bind Flt1 suggests that the binding motif will be different. We were unable to co-immunoprecipitate endogenous Shc with Flt when expressed in HEK-293 cells. However, addition of VEGF to the transfected cells did not promote phosphorylation of Flt1. It is not clear why Flt1 is so resistant to ligand-induced kinase activation, since when the cytoplasmic domain of Flt1 is expressed in insect cells [46] or in yeast (results not shown), it is constitutively phosphorylated. It is possible that Flt1 requires a co-receptor to enable it to become efficiently phosphorylated in response to VEGF, and this receptor is not expressed in most cell types. Recently, such a co-receptor, neuropilin 1, was reported for KDR [47]. Our inability to induce Flt1 phosphorylation with VEGF, and indeed P1GF, in transfected HEK-293 cells, PAE cells and primary HUVECs, has prevented us from studying the role of Shc or Sck further in this context. We have, however, recently established PAE cell lines expressing chimaeric VEGF receptors which can be induced to phosphorylate (E. L. Knight and S. A. Prigent, unpublished work), and these cell lines should assist us in establishing the *in vivo* significance of some of the interactions we, and others have detected in the yeast two-hybrid assay with Flt1.

While our studies were being completed, another group confirmed that, in the two-hybrid assay, Sck is able to bind to the receptor KDR, but did not extend these studies to demonstrate an association *in vivo* [48]. Those authors found that the binding of Sck to KDR is mediated through its SH2 domain, and although the PTB domain may stabilize association, it is not involved in direct interaction. They were unable to identify a binding site for Flt1, which may suggest that this particular interaction is rather non-specific, or that multiple sites exist for Shc and Sck binding on Flt1. These investigators did, however, confirm that Sck is indeed expressed in HUVECs, which adds support to the suggestion that it could be a physiological effector for KDR.

It seems likely that all Shc family members, Shc, Sck and N-Shc [49], will bind Grb2 when phosphorylated and couple to the Ras–MAP kinase cascade, since the Grb2-binding sites are completely conserved. While Trk and EGF receptors bound and phosphorylated Shc, Sck and N-Shc [39], c-Src was not able to efficiently phosphorylate Sck, but did phosphorylate N-Shc. Moreover, Sck was able to bind a phosphoprotein, pp135, which was phosphorylated by c-Src, whereas N-Shc was not [39]. These results suggest that different Shc family members may share common effector pathways as well as participating in distinct ones. It will be of importance to further define the roles of Shc and Sck in integrating the complex network of events triggered by VEGF binding to Flt1 and KDR. Identification of the Sck-binding site on KDR provides the first step towards elucidating the role of Sck in VEGF signalling, by examining the ability of KDR mutants lacking this site to promote MAP kinase activation, migration and proliferation of PAE cells in response to VEGF.

We are grateful to Lena Claesson-Welsh and Bruce Terman for allowing us to use the PAE/KDR cell line and to Stan Hollenberg for the use of the mouse embryo library. We also thank Richard May for performing some of the experiments. This work was funded in part by grants from the Wellcome Trust and the British Heart Foundation. S.A.P. is the recipient of a fellowship from the Lister Institute of Preventive Medicine.

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Received 5 October 1999/11 January 2000; accepted 7 February 2000

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