Direct identification of a major autophosphorylation site on vascular endothelial growth factor receptor Flt-1 that mediates phosphatidylinositol 3'-kinase binding

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Progress has been made in our understanding of the mechanism by which the binding of vascular endothelial growth factor (VEGF) to cognate receptors induces a range of biological responses, but it is far from complete. Identification of receptor autophosphorylation sites will allow us to determine how activated VEGF receptors are coupled to specific downstream signalling proteins. In the present study, we have expressed human VEGF receptors in insect cells using the baculovirus expression system, identified a major autophosphorylation site on the VEGF receptor fms-like tyrosine kinase-1 (Flt-1) by HPLC–electrospray ionization (ESI)–MS, and characterized *in vitro* interactions between Flt-1 and phosphatidylinositol 3'-kinase (PI3-kinase). Infection of High 5 insect cells with Flt-1 recombinant virus resulted in the expression of a 170 kDa glycoprotein, which bound VEGF with a K_d of 2×10^{-10} M in

intact insect cells. The overexpressed recombinant Flt-1 receptors exhibited tyrosine kinase activity and were constitutively phosphorylated. Analysis of Flt-1 tryptic peptides by HPLC–ESI–MS with selective phosphate ion monitoring identified a hexapeptide (YVNAFK; where single-letter amino-acid code has been used) containing a phosphotyrosine (pTyr) residue at position 1213. Using synthetic phosphopeptides, this pTyr residue was found to be directly involved in the binding of PI3-kinase *in vitro* even though it did not fall within a consensus pYM/VXM PI3-kinase binding motif. These results suggest that phosphorylated Flt-1 associates with PI3-kinase at pTyr¹²¹³ to mediate the activation of this pathway in VEGF signalling.

Key words: mass spectrometry, phosphotyrosine, receptor tyrosine kinase.

INTRODUCTION

The tyrosine kinase receptors fms-like tyrosine kinase-1 (Flt-1) and kinase insert domain-containing receptor [KDR; also known as fetal liver kinase-1 (Flk-1)], the two signal transmitting receptors for vascular endothelial growth factor (VEGF), play critical roles in angiogenesis. Gene targeting studies have shown that KDR/Flk-1 null mice lack blood islands and haematopoietic progenitor cells, while Flt-1 null mice have differentiated endothelial cells, but fail to form an organized embryonic vasculature [1,2]. These results indicated that Flt-1 and KDR/Flk-1 have distinct physiological roles during development and that both receptors are essential for survival.

Ligand binding activates receptor tyrosine kinases (RTKs) through receptor dimerization and autophosphorylation [3]. We demonstrated that VEGF activated a tyrosine kinase activity in human umbilical cord endothelial cells [4], and Flt-1 [5] and KDR [6] were subsequently characterized as VEGF-activated RTKs. Individual phosphotyrosine (pTyr) residues and adjacent amino acids generate highly selective binding sites for cytoplasmic signalling proteins containing Src homology (SH)2 or pTyr-binding ('PTB') domains [7]. Association of signalling proteins with phosphorylated receptors then results in the activation of intracellular signalling pathways, which induce gene expression and cellular responses [8]. Thus identification of VEGF receptor autophosphorylation sites will provide considerable insight into the interactions between VEGF receptors and downstream

signalling proteins, and lead to a better understanding of the functions of individual VEGF receptors in regulating endothelial cells. We have recently reported that the mitogen-activated protein kinase (MAP kinase) and phosphatidylinositol 3'-kinase (PI3-kinase)/p70 S6 kinase pathways are essential for VEGF-induced endothelial cell proliferation while the p38 MAP kinase pathway regulates VEGF mitogenic activity by suppressing cell cycle progression [9]. However, the specific protein–protein interactions through which VEGF receptors activate specific downstream signalling pathways to transduce a mitogenic signal are not clear. Dougher-Vermazen et al. [10] found four pTyr residues (pTyr⁹⁵¹, pTyr⁹⁹⁶, pTyr¹⁰⁵⁴ and pTyr¹⁰⁵⁹) in bacterially-expressed KDR kinase domain. Thus far, none of these pTyr residues has been specifically implicated in the binding of any signalling molecules, but KDR has been implicated in the activation of protein kinase B/Akt through PI3-kinase in endothelial cells [11], and experiments with the KDR inhibitor SU5416 suggest that KDR is involved in the activation of phospholipase C- γ (PLC- γ), protein kinase C- ϵ , and MAP kinase [12]. Several putative autophosphorylated tyrosine residues on Flt-1 were identified using either the yeast two-hybrid system or two-dimensional phospho amino acid analysis [13–17], but these reports made conflicting claims regarding phosphorylated tyrosine residues on Flt-1 and their potential binding proteins. Using the same technique, pTyr¹¹⁶⁹ was identified in full-length Flt-1 [15], but not in overexpressed Flt-1 intracellular domain [17]. PLC- γ was found by Cunningham et al. [14], and Sawano et al.

Abbreviations used: ESI, electrospray ionization; FBS, fetal bovine serum; Flk-1, fetal liver kinase-1; Flt-1, fms-like tyrosine kinase-1; HUVECs, human umbilical vein endothelial cells; KDR, kinase insert domain-containing receptor; MAP kinase, mitogen-activated protein kinase; PDGF, platelet-derived growth factor; PI3-kinase, phosphatidylinositol 3'-kinase; PLC- γ , phospholipase C- γ ; PNGase F, peptide N-glycosidase F; pTyr, phosphotyrosine; RTK, receptor tyrosine kinase; SH, Src homology; SIM, selective ion monitoring; VEGF, vascular endothelial growth factor.

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[15], to bind to pTyr¹¹⁶⁹; however, Ito et al. [17] reported that PLC- γ bound to pTyr¹²¹³ and pTyr¹³³³. Thus these experimental approaches have not yielded a consensus on either the identities of autophosphorylation sites or their interactions with signal transduction molecules. In the present study, we sought to identify autophosphorylated tyrosine residues on full-length recombinant human Flt-1 expressed in insect cells using a different technique, electrospray ionization (ESI)-MS, and to identify associated downstream signalling proteins.

ESI [18,19] was introduced as a novel way to volatilize peptides for mass analysis. When directly coupled to a chromatography system, on-line analysis of column eluates can be accomplished without the need for peptide purification. The use of triple quadrupole mass analysers allows the selection of parent ions of specific mass to charge ratio in the first quadrupole, which are then fragmented by collision-induced dissociation in the second quadrupole. A series of fragment ions that provide sequence information are measured in the third quadrupole. This process, known as tandem MS or MS-MS [20], can be applied to peptides in mixtures or in eluates from HPLC. Selective ion monitoring (SIM) monitors ion current at certain mass values throughout the complete mass spectrum as a function of time [21]. The combination of SIM with ESI-MS has been a powerful tool for identifying protein modifying groups at the post-translational level [22,23].

In the present study we report that Tyr¹²¹³ is a major autophosphorylation site on Flt-1. Using synthetic phosphopeptides, we also show that PI3-kinase physically associates with Flt-1 at pTyr¹²¹³. These results indicate that Flt-1 participates in the regulation of endothelial cell functions through PI3-kinase-activated signalling pathways, and they are consistent with our previous finding that PI3-kinase signalling through p70 S6 kinase is required for VEGF-induced human endothelial cell proliferation [9].

EXPERIMENTAL

Reagents

Grace's insect cell culture medium, lactalbumin hydrolysate and yeastolate were purchased from Life Technologies (Gaithersburg, MD, U.S.A.). Fetal bovine serum (FBS) was purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Sf9 and High 5 insect cells, and the pVL1392 baculovirus transfer vector were obtained from Invitrogen (Carlsbad, CA, U.S.A.). Anti-(Flt-1) (C-17) and anti-(Flk-1) (C-1158) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Monoclonal anti-pTyr antibody (4G10) and monoclonal anti-(PI3-kinase) (p85 α) were purchased from Upstate Biotechnology. Protein A-Sepharose, Protein G-agarose and Sulfolink coupling gel were from Pierce Chemical Co. (Rockford, IL, U.S.A.). PVDF membrane was from Millipore (Bedford, MA, U.S.A.), and ECL[®] Western blotting detection reagents were obtained from Amersham (Little Chalfont, Bucks., U.K.). [γ -³²P]ATP and ³⁵S-protein labelling mix were from NEN Life Science Products (Boston, MA, U.S.A.). Na₃VO₄ and the protease inhibitors leupeptin, pepstatin A, aprotinin and PMSF were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Acetonitrile and trifluoroacetic acid were obtained from Fisher Scientific (Springfield, NJ, U.S.A.).

Cell culture

Sf9 and High 5 insect cells were maintained at 27 °C in Grace's insect culture medium supplemented with lactalbumin hydrolysate, yeastolate and 10% (v/v) FBS. Human umbilical vein

endothelial cells (HUVECs) were isolated and maintained as previously described [9].

RNA isolation and reverse transcription

Total RNA was prepared from HUVECs using RNazol (Biotecx Laboratories, Houston, TX, U.S.A.). cDNA templates for PCR were synthesized from 5 μ g of total RNA with oligo(dT) primers using a SuperScript preamplification system (Life Technologies).

Cloning and sequencing of VEGF receptor cDNAs

cDNA fragments (1.6 kb) encoding the intracellular portion of Flt-1 or KDR were cloned using PCR. The following synthetic oligonucleotide primer pairs were used for Flt-1 (forward, 5'-CCTTATGATGCCAGCAAGTGG-3'; reverse, 5'-CTGGATCCGGCTTCGTGTCAAACCTCTAG-3') and KDR (forward, 5'-CATGGATCCAGATGAATCCCATTTGG-3'; reverse, 5'-CTGGATCCCTTAAACAGGAGGAGCTC-3'). Full-length receptor cDNAs were made by splicing the 1.6 kb Flt-1 fragment on to a 2.4 kb cDNA fragment encoding the extracellular portion of Flt-1 in the pVL1392 transfer vector [24] cut with the restriction enzymes *Pst*MI and *Bam*HI, and by splicing the 1.6 kb KDR fragment on to a 2.4 kb cDNA fragment encoding the extracellular portion of KDR in the pVL1392 vector [24] at a unique *Bam*HI site. Samples for sequence analysis were prepared using the Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (PE Biosystems, Foster City, CA, U.S.A.). The complete cDNAs of KDR and Flt-1 were sequenced on an Applied Biosystems Model 370A DNA sequencer.

Antibodies

Rabbit polyclonal antisera P20L and K20L were raised against the synthetic peptides PNNQSGSEQRVEVTECSDDL and KDP-ELSLKGTQHIMQAGQTL (where single-letter amino-acid code has been used) corresponding to N-terminal amino acid sequences of human KDR and Flt-1 respectively. The peptides were synthesized by the Adirondack Biomedical Research Institute Protein Chemistry Facility. Immunization of rabbits with keyhole-limpet haemocyanin-conjugated peptides and collection of sera were performed by Cocalico Biologicals (Reamstown, PA, U.S.A.). The antibodies were affinity-purified using peptide-coupled Affi-gel 10 (Bio-Rad).

Generation of recombinant baculovirus and infection of High 5 cells

Recombinant viruses were generated by co-transfection of Sf9 cells with PVL1392/Flt-1 or PVL1392/KDR and Baculogold baculovirus DNA (BD Pharmingen, San Diego, CA, U.S.A.) using Lipofectin (Life Technologies). Recombinant viruses were purified by plaque assay [25], and were verified by PCR. Cells were lysed and the recombinant proteins were analysed by immunoprecipitation and/or immunoblotting 48 h post-infection.

Metabolic labelling of recombinant Flt-1 and KDR

Subconfluent High 5 cells in a 24-well plate (Corning) were infected with recombinant viruses. Prior to labelling, the cells were incubated with methionine-deficient medium (Life Technologies) for 1 h. The same medium containing 25 μ Ci of ³⁵S-protein labelling mix (NEN Life Science Products) was added, 2 h before each time point. Incubation was continued at 27 °C

for 2 h with occasional gentle rocking. Cells were gently rinsed three times with PBS and harvested with a rubber policeman. The cells were collected by microcentrifugation, then lysed in buffer containing 0.1 % SDS, 1 % (v/v) Triton X-100, 10 µg/ml PMSF, 5 mM EDTA and 1 µg/ml leupeptin. Labelled proteins were analysed by SDS/PAGE and autoradiography.

Immunoprecipitation and immunoblotting of recombinant Flt-1 and KDR

Infected High 5 cells were washed twice with ice-cold PBS (pH 6.2), and then incubated with lysis buffer [50 mM Tris/HCl (pH 7.5), 1 % (v/v) Nonidet P40, 150 mM NaCl, 5 mM EDTA, 10 % (v/v) glycerol, 1 mM PMSF, 1 mM Na₃VO₄, 50 µg/ml leupeptin and 50 µg/ml aprotinin] at 4 °C for 20 min. The lysates were cleared by microcentrifugation at 12000 g for 20 min at 4 °C. Supernatants were incubated with 5 µg of K20L or P20L antibodies for 2–3 h at 4 °C, then 30 µl of a 50 % (v/v) Protein A–Sepharose (Pierce) slurry was added and incubation was continued for 1 h. The immune complexes were pelleted by centrifugation, and washed three times with lysis buffer and once with ice-cold PBS. Proteins were eluted by boiling at 95 °C for 5 min in 25 µl of reducing 1.5 × Laemmli sample buffer. Proteins were then resolved by SDS/PAGE [7.5 % (w/v) polyacrylamide], transferred on to a PVDF membrane and analysed by immunoblotting with polyclonal anti-(Flt-1) (C-17) or anti-(Flk-1) (C-1158) antibodies using ECL[®] (Amersham) detection reagents.

Ligand binding assay

The affinity of VEGF for recombinant Flt-1 expressed in High 5 cells was determined in a saturation binding assay as described previously [24].

In vitro tyrosine kinase assay

After immunoprecipitation, the immune complexes were washed three times with lysis buffer and twice with kinase buffer [50 mM Hepes (pH 7.4), 10 mM MnCl₂, 1 mM dithiothreitol, 0.1 mM Na₃VO₄ and 1 µg/ml leupeptin], and were subsequently incubated in 25 µl of kinase buffer containing 5 µCi of [γ -³²P]ATP at 25 °C for 10 min. The reactions were terminated by adding 6 × Laemmli sample buffer. The samples were resolved by SDS/PAGE [7.5 % (w/v) polyacrylamide] and transferred to a PVDF membrane. The membrane was treated for 2 h at 55 °C in 1 M KOH to remove serine- or threonine-bound phosphate [26], and was subsequently subjected to autoradiography. Membrane-bound receptors were detected by immunoblotting.

Preparation of membranes from High 5 cells expressing Flt-1

High 5 cells were gently scraped in ice-cold PBS (pH 6.2) containing 0.1 M PMSF, and were collected by centrifugation at 1000 g at 4 °C for 5 min. The cells were then disrupted by homogenization in ice-cold buffer containing 20 mM Tris/HCl (pH 7.5), 2 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM Na₃VO₄, 10 µg/ml leupeptin, 10 µg/ml aprotinin and 1 µg/ml pepstatin A. The homogenates were centrifuged at 1000 g for 10 min at 4 °C. The pellet was homogenized again, and the supernatants were pooled and then centrifuged at 30000 g for 30 min. The pellet was resuspended in the same buffer and centrifuged at 100000 g for 1 h at 4 °C. The membrane pellet was resuspended at a protein concentration of 10 mg/ml in solubilization buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2 mM PMSF, 2 mM Na₃VO₄, 10 µg/ml leupeptin, 10 µg/ml aprotinin and 1 µg/ml pepstatin A] and stored at

–70 °C. The protein was solubilized in Laemmli sample buffer and resolved by SDS/PAGE [7.5 % (w/v) polyacrylamide]. The gel was divided in two: one part was stained with Coomassie Brilliant Blue (Bio-Rad), while the other was transferred on to a PVDF membrane and probed with receptor-specific antibodies. Receptor bands were cut from the stained gel and subjected to HPLC–ESI–MS to identify pTyr residues.

Identification of autophosphorylation sites on recombinant Flt-1

The excised recombinant Flt-1 bands were cut into gel pieces (1–2 mm³), washed and dehydrated with 50 % (v/v) acetonitrile containing 0.2 M NH₄HCO₃. Proteins were then digested in-gel with trypsin. In each reaction, approx. 4.5–9 µg of Flt-1 was incubated with 1.67 µg of trypsin in 0.1 M NH₄HCO₃ at 37 °C for 16 h. Digested peptides were extracted with 60 % (v/v) acetonitrile and 0.2 % trifluoroacetic acid. The peptides were then separated and analysed by ESI–MS, HPLC–ESI–MS with selective phosphate ion monitoring and low energy collision tandem MS using a PerkinElmer Sciex API 300 triple quadrupole mass spectrometer (PE Biosystems) [27]. The Bio MultiView data processing program (PE Biosystems) was used for data analysis.

Immobilization of synthetic peptides

Peptides CG15F (CGSSDDVRYVNAFKF) and CG15F-pY (CGSSDDVRpYVNAFKF) were synthesized by Adirondack Biomedical Research Institute Protein Chemistry Facility. Cysteine at the N-termini of peptides was used to conjugate peptides to Sulfolink coupling gel (Pierce Chemical Co.) according to the manufacturer's instructions. Briefly, 0.4 mg of synthetic peptide was dissolved in coupling buffer [50 mM Tris/HCl (pH 8.5) containing 5 mM EDTA], and mixed with 0.8 ml of Sulfolink gel equilibrated with the same buffer. Non-specific binding sites on the gel were blocked by 1 ml of 50 mM L-cysteine/HCl. The gel was further washed with 1 M NaCl and 0.05 % sodium azide, and stored in degassed deionized water containing 0.05 % sodium azide at 4 °C.

In vitro binding of autophosphorylated recombinant Flt-1 to PI3-kinase

Recombinant Flt-1 was immunoprecipitated from infected High 5 cells as described above. The immune complexes were washed once with lysis buffer containing 0.1 % SDS, twice with lysis buffer, and once with 10 mM Tris/HCl (pH 7.4). Recombinant Flt-1 was further phosphorylated by incubating in kinase buffer [30 mM Tris/HCl (pH 7.4), 10 mM MnCl₂, 50 µM ATP, 0.1 mM Na₃VO₄ and 1 µg/ml leupeptin] at 25 °C for 15 min. The complexes were washed twice with lysis buffer, and then incubated with 1 mg of HUVEC lysates at 4 °C for 2 h. The HUVEC lysates were preincubated with CG15F or CG15F-pY peptides at 4 °C for 30 min. After being washed four times in lysis buffer and once in 10 mM Tris/HCl (pH 7.4), Flt-1-bound proteins were eluted by boiling at 95 °C for 5 min in 35 µl of reducing 1.5 × Laemmli sample buffer. Proteins were then resolved by SDS/PAGE [7.5 % (w/v) polyacrylamide] and detected by immunoblotting with monoclonal anti-(PI3-kinase) or polyclonal anti-(Flt-1) (C-17) antibodies.

In vitro binding of immobilized peptides with PI3-kinase

Unstimulated HUVECs were lysed in 50 mM Tris/HCl (pH 7.5), 1 % (v/v) Nonidet P40, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 mM Na₃VO₄, 1 mM PMSF, 10 mM NaF, 10 µg/ml

leupeptin, 10 $\mu\text{g}/\text{ml}$ aprotinin and 1 $\mu\text{g}/\text{ml}$ pepstatin. Cell lysates were clarified by centrifugation twice at 13000 g for 15 min. A 50 % (v/v) peptide–Sulfolink gel slurry (30 μl) containing 3 μg of peptide was incubated with 0.5 mg of HUVEC lysate protein at 4 °C for 2 h. The gel beads were washed with lysis buffer containing 0.2 M NaCl, and the bound proteins were analysed by Western blotting with a monoclonal antibody directed against PI3-kinase.

RESULTS

Molecular cloning of Flt-1 cDNA

The full-length coding region cDNA for Flt-1 was amplified from HUVEC RNA by reverse transcription PCR using Taq and Vent polymerases, and it was subcloned into the baculovirus transfer vector PVL1392. Two nucleotide differences were found between our Flt-1 sequence and the original published sequence [28], resulting in a single amino acid difference (Leu⁷⁷⁹ → Phe) that was also reported by de Vries et al. [5]. For comparison KDR coding region cDNA was also cloned and sequenced. The Flt-1 and KDR cDNA sequences have been deposited with GenBank® (accession numbers AF063657 and AF063658).

Recombinant Flt-1 as a glycosylated membrane-bound protein with specific binding affinity for VEGF

Recombinant Flt-1 expressed in insect cells was detected in cell lysates by Western blotting as a single protein of approx. 170 kDa, whereas recombinant KDR was detected as a doublet of approx. 190 and 170 kDa (Figure 1). To determine whether recombinant Flt-1 was post-translationally glycosylated, similarly to endogenous Flt-1 in endothelial cells, membrane proteins prepared from infected High 5 cells were digested with peptide N-glycosidase F (PNGase F). Recombinant Flt-1 was essentially completely digested by this enzyme, reducing the apparent molecular mass estimated by SDS/PAGE from 170 to 155 kDa, which was close to the 150 kDa mass predicted for the non-glycosylated Flt-1

Figure 1 Time course of Flt-1 and KDR expression in baculovirus-infected High 5 cells

(A) Autoradiogram of ³⁵S-labelled proteins of cells that were pulse-labelled for 2 h and lysed at the indicated time points. Detergent lysates were resolved by SDS/PAGE, and the dried gel was exposed to X-ray film. (B) Flt-1 and KDR were immunoprecipitated (IP) from detergent lysates at 0, 24, 36 and 48 h post-infection, and were subjected to SDS/PAGE. They were then detected by immunoblotting (IB). The antibodies (Ab) used are listed. Molecular masses (kDa) of protein standards are indicated on the left-hand sides of the panels.

Figure 2 PNGase F digestion of Flt-1 and KDR expressed in High 5 insect cells

Recombinant virus-infected High 5 (H5) cells were lysed 48 h post-infection. Cells were fractionated by homogenization, and membranes were collected by ultracentrifugation. Digestion was carried out by incubating membrane proteins with (+) or without (–) PNGase F at 37 °C overnight. Flt-1 or KDR in the digestion mixture was analysed by Western blotting with the indicated anti-peptide antibodies. Molecular masses (kDa) of protein standards are indicated on the left-hand side.

Figure 3 Analysis of VEGF binding to intact High 5 cells expressing Flt-1

Scatchard plot of the saturation binding assay (shown in the inset). Increasing amounts of [¹²⁵I]VEGF were added to Flt-1 virus-infected High 5 cells that had been fixed in 48 well plates. Non-specific binding was determined by competition with a 100-fold excess of unlabelled VEGF. After binding, the cells were washed and lysed, and the cell-associated radioactivity was determined using a gamma counter. Results are presented as the means of triplicate determinations.

polypeptide (Figure 2). Recombinant KDR was similarly deglycosylated by PNGase F. It is noteworthy that in cell membranes, KDR was present as a single 190 kDa protein, which corresponded to the higher molecular mass KDR polypeptide detected in whole-cell lysates (Figure 1). When KDR-infected High 5 cells were incubated in the presence of the glycosylation inhibitor tunicamycin, only the lower molecular mass KDR was expressed (results not shown), indicating that the 170 kDa KDR was not glycosylated. Recombinant Flt-1 bound to the 165-amino-acid isoform of VEGF in a saturable manner (Figure 3).

Figure 4 Ligand-independent VEGF receptor tyrosine phosphorylation in Flt-1- or KDR-infected High 5 (H5) cells

Left-hand panel: at 48 h post-infection, cells were treated with (+) or without (–) 50 ng/ml VEGF for 5 min. Flt-1 or KDR was immunoprecipitated (IP) from whole-cell lysates with anti-peptide antibodies K20L or P20L, and analysed by Western blotting with anti-pTyr antibodies. Molecular masses (kDa) of protein standards are indicated on the left-hand side. Right-hand panel: Flt-1 or KDR immunoprecipitates were subjected to an *in vitro* kinase assay. Samples were analysed by SDS/PAGE, and transferred on to a PVDF membrane, followed by KOH treatment and autoradiography. Flt-1 or KDR was detected on the membrane by immunoblotting (IB) with anti-Flt-1 (C-17) or anti-KDR (C-1158) antibodies.

Scatchard analysis revealed the presence of a single class of high-affinity binding sites on these cells with a dissociation constant (K_d) of 2×10^{-10} M, indicating that the recombinant Flt-1 was intact and functional.

Ligand-independent tyrosine phosphorylation of recombinant Flt-1

Western blotting with an anti-pTyr antibody of anti-receptor immunoprecipitates of infected cells treated with or without VEGF indicated that both Flt-1 and KDR were tyrosine phosphorylated in the absence of VEGF (Figure 4, left-hand panel). These phosphorylation events resulted from receptor autophosphorylation and not from the activity of unrelated tyrosine kinases as demonstrated recently by Sawano et al. [15] with an Flt-1 kinase-negative mutant, which exhibited no tyrosine phosphorylation in insect cells. Ligand-independent autophosphorylation of RTKs has been previously reported for platelet-derived growth factor (PDGF) receptors [29,30], epidermal growth factor receptors [31,32] and fibroblast growth factor receptors [33] expressed in the baculovirus system. The intrinsic kinase activity of recombinant Flt-1 or KDR was confirmed in an *in vitro* kinase assay (Figure 4, right-hand panel). These results indicated that the baculovirus system was a useful model with which to study the autophosphorylation sites of the Flt-1 receptor, whose tyrosine kinase activity is difficult to detect in a mammalian cell environment [34,35].

Identification of autophosphorylation sites on Flt-1

To produce sufficient amounts of tyrosine autophosphorylated recombinant Flt-1, we initially tried to purify Flt-1 from insect cells by affinity chromatography on a VEGF column. Flt-1 receptors were first enriched by isolating plasma membranes from infected High 5 cells pretreated with VEGF. In tests with a variety of detergents, the efficiency of solubilizing Flt-1 from the membranes, however, was extremely low. We then took an alternative approach and purified Flt-1 by SDS/PAGE. Membrane-derived 170 kDa Flt-1 protein was clearly visualized by Coomassie Brilliant Blue on an SDS/polyacrylamide gel (Figure

Figure 5 Preparation of baculovirus-expressed recombinant Flt-1 for analysis by MS

Flt-1-infected High 5 cells (48 h post-infection) were fractionated by homogenization and ultracentrifugation. Membrane and cytosolic proteins were resolved by SDS/PAGE [7.5% (w/v) polyacrylamide]. Flt-1 was located in the gel stained with Coomassie Brilliant Blue (top panel). A parallel gel (bottom panel) was transferred on to a PVDF membrane and immunoblotted with anti-Flt-1 (C-17) antibodies. The immunoreactive Flt-1 corresponds to a 170 kDa band in the stained gel. Flt-1 protein bands were excised from the stained gel, digested with trypsin and analysed using an API 300 HPLC–tandem MS mass spectrometer.

5, top panel). In a parallel gel, this 170 kDa protein was recognized by anti-Flt-1 antibodies (Figure 5, bottom panel) and by an anti-pTyr antibody (results not shown). After trypsin digestion of Flt-1 protein bands excised from the stained gel, tryptic peptide extracts were run on HPLC–MS.

The tryptic digests were analysed by capillary liquid chromatography coupled with ESI–MS. Flt-1 peptides were identified in HPLC–MS chromatograms by comparing observed masses with those of predicted proteolytic fragments. Peptide sequences were confirmed by tandem MS. Phosphorylated peptides were identified by SIM, in which phosphate groups were detected in the first quadrupole of the mass spectrometer as a result of partial dephosphorylation. This was achieved by applying a high orifice potential to a portion of each sample. pTyr-containing peptides were then detected where phosphate ions (PO_3^- in negative ion mode) were identified by mass increases of 80 Da. The only phosphopeptide detected was the tryptic hexapeptide YVNAFK (Figure 6), which contained a phosphorylated tyrosine residue (pTyr¹²¹³) derived from a region near the C-terminus of Flt-1.

Flt-1 interaction with PI3-kinase is determined by Tyr¹²¹³

The three amino acids immediately C-terminal to an RTK pTyr residue define the binding specificity for SH2 domain-containing proteins [8,36]. The phosphopeptide pYVNA of Flt-1 fits the general binding motif pTyr-hydrophobic-Xaa-hydrophobic elucidated for PI3-kinase, PLC- γ and SH protein-tyrosine phosphatase 2 ('SHPTP2') [36]. Cunningham et al. [13] inferred that Flt-1 bound PI3-kinase through pTyr¹²¹³ from results obtained with Flt-1 kinase domain and PI3-kinase SH2 domain constructs in the yeast two-hybrid system. In addition, we have reported that VEGF activated the PI3-kinase pathway in human endothelial cell mitogenesis [9]. From these results, PI3-kinase appeared to be a potential signalling protein that could be associated with autophosphorylated Flt-1.

Figure 6 HPLC-ESI-MS analysis of tryptic Flt-1 VEGF receptor

Approx. 10 pmol of an Flt-1 tryptic digest was analysed by HPLC-ESI-MS with SIM for phosphopeptides (PO_3^- in negative ion mode). (A–C) Are from one run using a 140D capillary HPLC system (Applied Biosystems) and a 0.5 mm $\times$ 150 mm C_{18} column (Applied Biosystems) at a flow rate of 5 $\mu\text{l}/\text{min}$. *Biotin-ApSSYK (10 pmol) and EPQpYEEIPIA- NH_2 (4 pmol) were injected with the digestion mixture as internal positive control phosphopeptides. All of the column effluent was analysed with an API 300 ESI mass spectrometer. (A) UV absorbance profile at 210 nm. (B) Negative ion detection profile for PO_3^- . (C) Full scan positive total ion current (TIC). The pTyr-containing hexapeptide pYVNAFK is indicated. $6.0\text{e}6 = 6.0 \times 10^6$.

To demonstrate a direct physical interaction between Flt-1 and PI3-kinase, with the involvement of pTyr¹²¹³, *in vitro* binding studies were performed. Two 15-residue peptides corresponding to Flt-1 amino acid residues 1206–1219 were synthesized, in which Tyr¹²¹³ was phosphorylated (CG15FpY) or left unmodified (CG15F) (see Figure 7A). To determine whether autophosphorylated recombinant Flt-1 associated with mammalian PI3-kinase, Flt-1 immunoprecipitates from infected High 5 cells were extensively washed and incubated with lysates from non-stimulated HUVECs in the presence of CG15F or CG15FpY peptides (Figure 7B, right-hand panel). Western-blot analysis of bound proteins with a specific antibody raised against PI3-kinase revealed that recombinant full-length Flt-1 associated with PI3-kinase, and the binding was completely blocked by the phosphopeptide CG15FpY, but not by the control peptide CG15F. Unrelated pTyr-containing peptides of similar length also had no effect on the co-immunoprecipitation of Flt-1 and PI3-kinase (results not shown). These results indicated that the interaction with PI3-kinase was dependent on the phosphorylation of Tyr¹²¹³. The requirement of pTyr¹²¹³ for the binding of PI3-kinase was also investigated using immobilized phosphopeptides. CG15F and CG15FpY were covalently coupled to Sulfolink agarose gel through their free N-terminal cysteine residues, and they were incubated with HUVEC lysates. The peptide-coupled Sulfolink gel was then collected by centrifugation and washed, and bound proteins were analysed by Western blotting with an anti-(PI3-kinase) antibody. As shown in Figure 7(B) (left-hand panel), the

Figure 7 *In vitro* association of PI3-kinase with Flt-1 receptors at pTyr¹²¹³

(A) The location of Tyr¹²¹³ in Flt-1 and the sequences of synthetic peptides CG15F and CG15FpY. The two hatched boxes represent the split tyrosine kinase domain of the Flt-1 receptor. (B) Right-hand panel: Recombinant Flt-1 receptors were immunoprecipitated from infected High 5 cells at 48 h post-infection with specific antibodies (K20L) to Flt-1 and incubated with cell lysates of non-stimulated HUVECs in the presence of peptide CG15F or CG15FpY. Bound proteins were analysed by Western blotting with a monoclonal anti-(PI3-kinase) antibody. The position of PI3-kinase is marked with an arrow. Left-hand panel: HUVEC lysates were incubated with the peptides coupled to Sulfolink gel in the absence (—) or presence (+) of 40 mM phenylphosphate. Bound proteins were analysed by Western blotting.

CG15FpY-coupled gel bound PI3-kinase, whereas the non-phosphorylated control peptide exhibited no detectable binding to PI3-kinase. Furthermore, the association of the immobilized phosphopeptide with the PI3-kinase was abolished when phenylphosphate was added to the incubation, indicating that the protein–protein interaction was mediated via pTyr¹²¹³. Western blotting also showed that the CG15FpY phosphopeptide bound PLC- γ (results not shown), which is in agreement with the results of Ito et al. [17]. Our data demonstrate that PI3-kinase binds directly to Flt-1, and this interaction is mediated specifically by pTyr¹²¹³.

DISCUSSION

A role for PI3-kinase in VEGF-induced cell signalling has been controversial. Although PI3-kinase is activated by a number of polypeptide growth factors [37], previous investigations found that PI3-kinase was not activated by VEGF in VEGF receptor cDNA-transfected porcine endothelial cells [34], Flt-1 cDNA-transfected NIH 3T3 fibroblasts [35], HUVECs [38], sinusoidal endothelial cells [39], or mouse capillary endothelial cells [17]. In support of a role for PI3-kinase in VEGF signalling, the p85 subunit of PI3-kinase was phosphorylated in bovine endothelial cells in response to VEGF [40], and it bound to pTyr¹²¹³ in the intracellular domain of Flt-1 in a yeast two-hybrid assay [13]. Subsequently, VEGF-stimulated PI3-kinase activity was shown to be important in the anti-apoptotic [11] and the proliferative [9,41] responses of human endothelial cells to VEGF. The present study provides additional evidence that PI3-kinase could mediate VEGF signalling through a direct physical interaction with activated Flt-1.

We have used HPLC-ESI-MS to identify Tyr¹²¹³ as the major autophosphorylation site on Flt-1. Previously, two different approaches were taken to identify Flt-1 phosphorylation sites. Using the yeast two-hybrid method coupled with site-directed mutagenesis, Cunningham et al. [13,14] identified Tyr¹²¹³, Tyr¹¹⁶⁹ and Tyr⁷⁹⁴ as residues modified by phosphorylation, whereas Igarashi et al. [16] identified Tyr¹²¹³ and Tyr¹³³³ as phosphorylation sites. Phospho amino acid analysis of wild-type and mutant tryptic peptides separated by two-dimensional gel electrophoresis indicated that Tyr¹²¹³ and Tyr¹¹⁶⁹ were autophosphorylated on full-length Flt-1 expressed in insect cells [15]. However, using the same method Ito et al. [17] reported four phosphorylation sites, Tyr¹²¹³, Tyr¹²⁴², Tyr¹³²⁷ and Tyr¹³³³, but not Tyr¹¹⁶⁹, on the baculovirus expressed Flt-1 kinase domain. Possible explanations for these discrepancies are differences in experimental conditions, assay sensitivities and receptor regions expressed (i.e. full-length receptor or tyrosine kinase domain). In each of these reports Flt-1 phosphorylation sites were identified by indirect methods. In the present study we have used MS to identify autophosphorylated Tyr¹²¹³ on recombinant full-length Flt-1 protein. No other phosphorylated tryptic peptides were detected. We cannot exclude the possibility that the phosphorylation levels of other Flt-1 tyrosine residues expressed in High 5 cells were below the detection limit of the MS system used. Although Tyr¹²¹³ was previously identified as a putative autophosphorylation site of Flt-1 that binds PI3-kinase [13,16], no direct evidence for this interaction was provided. In the present study we demonstrated an interaction between PI3-kinase and Tyr¹²¹³ with *in vitro* binding experiments using a synthetic phosphopeptide containing pTyr¹²¹³. The same phosphopeptide sequence also bound PLC- γ as shown by Ito et al. [17] (results not shown). PI3-kinase is composed of a p85 regulatory subunit, which contains two SH2 domains and one SH3 domain, and the p110 catalytic domain [42]. Since VEGF stimulates the phosphorylation of the p85 subunit of PI3-kinase in HUVECs [41] and induces kinase activity [9,43], the binding of inactive PI3-kinase to Flt-1 is most likely mediated by the SH2 domains of the p85 subunit as suggested by Cunningham et al. [13]. The YVNA sequence of Flt-1 that includes Tyr¹²¹³ is different from the YM/VXM motif for PI3-kinase that has been identified in other related RTKs, including the PDGF receptor, the colony-stimulating factor-1 receptor and c-kit. Thus there is more variability in the RTK binding motif recognized by PI3-kinase than previously appreciated.

We have previously shown that VEGF-activated PI3-kinase in HUVECs phosphorylates phosphatidylinositol, and this kinase plays an essential role in transducing a VEGF mitogenic signal [9]. The results reported in the present study suggest that the binding of activated Flt-1 through pTyr¹²¹³ to PI3-kinase contributes to the activation of PI3-kinase. This protein-protein interaction therefore couples an extracellular VEGF stimulus to the intracellular PI3-kinase signal transduction pathway in vascular endothelial cells.

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REFERENCES

- Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X. F., Breitman, M. L. and Schuh, A. C. (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature (London)* **376**, 62–66
- Fong, G. H., Rossant, J., Gertsenstein, M. and Breitman, M. L. (1995) Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature (London)* **376**, 66–70
- Heldin, C.-H. (1995) Dimerization of cell surface receptors in signal transduction. *Cell (Cambridge, Mass.)* **80**, 213–223
- Myoken, Y., Kayada, Y., Okamoto, T., Kan, M., Sato, G. H. and Sato, J. D. (1991) Vascular endothelial cell growth factor (VEGF) produced by A-431 human epidermoid carcinoma cells and identification of VEGF membrane binding sites. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5819–5823
- de Vries, C., Escobedo, J. A., Houck, K., Ferrara, N. and Williams, L. T. (1992) The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science (Washington, D.C.)* **255**, 989–991
- Terman, B. I., Dougher-Vermazen, M., Carrion, M. E., Dimitrov, D., Armellino, D. C., Gospodarowicz, D. and Bohlen, P. (1992) Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. *Biochem. Biophys. Res. Commun.* **187**, 1579–1586
- Kavanaugh, W. M., Turck, C. W. and Williams, L. T. (1995) PTB domain binding to signaling proteins through a sequence motif containing phosphotyrosine. *Science (Washington, D.C.)* **268**, 1177–1179
- Fantl, W. J., Johnson, D. E. and Williams, L. T. (1993) Signaling by receptor tyrosine kinases. *Annu. Rev. Biochem.* **62**, 453–481
- Yu, Y. and Sato, J. D. (1999) MAP kinases, phosphatidylinositol 3-kinase, and p70 S6 kinase mediate the mitogenic response of human endothelial cells to vascular endothelial growth factor. *J. Cell. Physiol.* **178**, 235–246
- Dougher-Vermazen, M., Hulmes, J. D., Bohlen, P. and Terman, B. I. (1994) Biological activity and phosphorylation sites of the bacterially expressed cytosolic domain of the KDR VEGF-receptor. *Biochem. Biophys. Res. Commun.* **205**, 728–738
- 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. *J. Biol. Chem.* **273**, 30336–30343
- 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
- Cunningham, S. A., Waxham, M. N., Arrate, P. M. and Brock, T. A. (1995) Interaction of the Flt-1 tyrosine kinase receptor with the p85 subunit of phosphatidylinositol 3-kinase. Mapping of a novel site involved in binding. *J. Biol. Chem.* **270**, 20254–20257
- Cunningham, S. A., Arrate, M. P., Brock, T. A. and Waxham, M. N. (1997) Interactions of FLT-1 and KDR with phospholipase C- γ : identification of the phosphotyrosine binding sites. *Biochem. Biophys. Res. Commun.* **240**, 635–639
- Sawano, A., Takahashi, T., Yamaguchi, S. and Shibuya, M. (1997) The phosphorylated 1169-tyrosine containing region of Flt-1 kinase (VEGFR-1) is a major binding site for PLC- γ . *Biochem. Biophys. Res. Commun.* **238**, 487–491
- Igarashi, K., Isohara, T., Kato, T., Shigeta, K., Yamano, T. and Uno, I. (1998) Tyrosine 1213 of Flt-1 is a major binding site of Nck and SHP-2. *Biochem. Biophys. Res. Commun.* **246**, 95–99
- Ito, N., Wernstedt, C., Engstrom, U. and Claesson-Welsh, L. (1998) Identification of vascular endothelial growth factor receptor-1 tyrosine phosphorylation sites and binding of SH2 domain-containing molecules. *J. Biol. Chem.* **273**, 23410–23418
- Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F. and Whitehouse, C. M. (1989) Electrospray ionization for mass spectrometry of large biomolecules. *Science (Washington, D.C.)* **246**, 64–71
- Edmonds, C. G. and Smith, R. D. (1990) Electrospray ionization mass spectrometry. *Methods Enzymol.* **193**, 412–431
- Yost, R. A. and Boyd, R. K. (1990) Tandem mass spectrometry: quadrupole and hybrid instruments. *Methods Enzymol.* **193**, 154–200
- Watson, J. T. (1990) Selected-ion measurements. *Methods Enzymol.* **193**, 86–106
- Ding, J., Burkhart, W. and Kassel, D. B. (1994) Identification of phosphorylated peptides from complex mixtures using negative-ion orifice-potential stepping and capillary liquid chromatography/electrospray ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **8**, 94–98
- Townsend, R. R., Lipniunas, P. H., Tuik, B. M. and Verkman, A. S. (1996) Identification of protein kinase A phosphorylation sites on NBD1 and R domains of CFTR using electrospray mass spectrometry with selective phosphate ion monitoring. *Protein Sci.* **5**, 1865–1873
- Herley, M. T., Yu, Y., Whitney, R. G. and Sato, J. D. (1999) Characterization of the VEGF binding site on the Flt-1 receptor. *Biochem. Biophys. Res. Commun.* **262**, 731–738
- O'Reilly, D. R., Miller, L. K. and Luckow, V. A. (1992) *Baculovirus Expression Vector: A Laboratory Manual*, CRC Press, Boca Raton, Florida

- 26 Kamps, M. P. and Sefton, B. M. (1989) Acid and base hydrolysis of phosphoproteins bound to immobilized facilitates analysis of phosphoamino acids in gel-fractionated proteins. *Anal. Biochem.* **176**, 22–27
- 27 Chapline, C., Cottom, J., Tobin, H., Hulmes, J., Crabb, J. and Jaken, S. (1998) A major, transformation-sensitive PKC-binding protein is also a PKC substrate involved in cytoskeletal remodeling. *J. Biol. Chem.* **273**, 19482–19489
- 28 Shibuya, M., Yamaguchi, S., Yamane, A., Ikeda, T., Tojo, A., Matsushima, H. and Sato, M. (1990) Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family. *Oncogene* **5**, 519–524
- 29 Jensen, R. A., Beeler, J. F., Heidaran, M. A. and LaRochelle, W. J. (1992) Characterization of baculovirus-expressed human α and β platelet-derived growth factor receptors. *Biochemistry* **31**, 10887–10892
- 30 Roche, S., Dhand, R., Waterfield, M. D. and Courtneidge, S. A. (1994) The catalytic subunit of phosphatidylinositol 3-kinase is a substrate for the activated platelet-derived growth factor receptor, but not for middle-T antigen-pp60^{c-src} complexes. *Biochem. J.* **301**, 703–711
- 31 Greenfield, C., Patel, G., Clark, S., Jones, N. and Waterfield, M. D. (1988) Expression of the human EGF receptor with ligand-stimulatable kinase activity in insect cells using a baculovirus vector. *EMBO J.* **7**, 139–146
- 32 Meisenhelder, J. and Hunter, T. (1992) The SH2/SH3 domain-containing protein Nck is recognized by certain anti-phospholipase C- γ 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
- 33 Shi, E., Kan, M., Xu, J., Wang, F., Hou, J. and McKehehan, W. L. (1993) Control of fibroblast growth factor receptor kinase signal transduction by heterodimerization of combinatorial splice variants. *Mol. Cell. Biol.* **13**, 3907–3918
- 34 Waltenberger, J., Claesson-Welsh, L., Siegbahn, A., Shibuya, M. and Heldin, C.-H. (1994) Different signal transduction properties of KDR and Flt-1, two receptors for vascular endothelial growth factor. *J. Biol. Chem.* **269**, 26988–26995
- 35 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
- 36 Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnoffsky, S., Lechleider, R. J. et al. (1993) SH2 domains recognize specific phosphopeptide sequences. *Cell (Cambridge, Mass.)* **72**, 767–778
- 37 Roche, S., Koegl, M. and Courtneidge, S. A. (1994) The phosphatidylinositol 3-kinase α is required for DNA synthesis induced by some, but not all, growth factors. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 9185–9189
- 38 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
- 39 Takahashi, T. and Shibuya, M. (1997) The 230 kDa mature form of KDR/Flk-1 (VEGF receptor-2) activates the PLC- γ pathway and partially induces mitotic signals in NIH3T3 fibroblasts. *Oncogene* **14**, 2079–2089
- 40 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**, 6729–6733
- 41 Thakker, G. D., Hajjar, D. P., Muller, W. A. and Rosengart, T. K. (1999) The role of phosphatidylinositol 3-kinase in vascular endothelial growth factor signaling. *J. Biol. Chem.* **274**, 10002–10007
- 42 Marengere, L. E. M. and Pawson, T. (1994) Structure and function of SH2 domains. *J. Cell Sci. Suppl.* **18**, 97–104
- 43 Gille, H., Kowalski, J., Li, B., LeCouter, J., Moffat, B., Zionscheck, T. F., Pelletier, N. and Ferrera, N. (2001) Analysis of biological effects and signaling properties of Flt-1 (VEGFR-1) and KDR (VEGFR-2). A reassessment using novel receptor-specific vascular endothelial growth factor mutants. *J. Biol. Chem.* **276**, 3222–3230

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