# Src mediates stimulation by vascular endothelial growth factor of the phosphorylation of focal adhesion kinase at tyrosine 861, and migration and anti-apoptosis in endothelial cells

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Vascular endothelial growth factor (VEGF) stimulates the tyrosine phosphorylation of focal adhesion kinase (FAK), increases focal adhesion formation and is chemotactic for human umbilical-vein endothelial cells (HUVECs). In the present study we identified the major sites of VEGF-induced FAK tyrosine phosphorylation and investigated the mechanism mediating this pathway in the action of VEGF. VEGF increased the focal adhesion localization of FAK phosphorylated at Tyr-397 (Y397) and Y861 but stimulated a marked increase in phosphorylation at Y861 without significantly affecting the total level of phospho-Y397 FAK. Inhibition of Src with the specific inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) completely blocked VEGF-induced Y861 phosphorylation without decreasing the level of phospho-Y397 FAK. We also examined the role of Src in mediating endothelial functions of VEGF in which FAK has been implicated as having a role. PP2 markedly inhibited VEGF-induced chemotaxis and wound-healing cell migration. The Src inhibitor also decreased the anti-apoptotic effect of VEGF determined by surface staining of annexin V but did not increase FAK proteolysis or prevent the VEGF-dependent inhibition of FAK proteolysis. In contrast, the specific PtdIns 3-kinase inhibitor LY294002 induced apoptosis and markedly decreased $p125^{FAK}$ expression and increased FAK proteolysis but had little effect on Y861 phosphorylation. These findings identify Src-dependent FAK phosphorylation at Y861 as a novel VEGF-induced signalling pathway in endothelial cells and suggest that this pathway might be involved in the mechanisms mediating VEGF-induced endothelial cell migration and anti-apoptosis.

Key words: chemotaxis, endothelium, KDR, survival.

## INTRODUCTION

Vascular endothelial growth factor (VEGF) is essential for endothelial cell differentiation (vasculogenesis) and angiogenesis during development of the embryonic vasculature and has a major role in angiogenesis in a variety of disease states [1–3]. VEGF expression is up-regulated by hypoxia and several cytokines [4–6] and elicits an array of biological activities *in vivo* and *in vitro*, including the survival, proliferation and migration of endothelial cells [7–9], the endothelial production of NO and prostaglandin $I_2$ ($PGI_2$) [10–13] and increased vascular permeability [14].

Two protein tyrosine kinase receptors for VEGF, KDR/Flk-1 and Flt-1, are essential for embryonic vascular development [15]. The biological effects of VEGF in endothelia are mediated primarily via KDR [16], whereas recent findings indicate that Flt-1 might act as a negative regulator of KDR [17]. Neuropilin-1 is a non-tyrosine kinase receptor for VEGF which might function as a 'docking' co-receptor for KDR [18]. After binding to KDR, VEGF activates several early signalling cascades in endothelial cells, including phospholipase C-$\gamma$, leading to increased activity of protein kinase C (PKC) and the mobilization of intracellular $Ca^{2+}$ via the production of Ins(1,4,5)$P_3$, the activation of p42/p44 extracellular signal-regulated protein kinases (ERKs) 1 and 2 and phosphoinositide 3-kinase (PI 3-kinase)-dependent Akt/protein kinase B activity [12,13,15,16, 19,20]. VEGF also stimulates the tyrosine phosphorylation of several intracellular components, including p125 focal adhesion kinase (FAK) and paxillin [21,22]. The biological function(s) of signal transduction mechanisms mediated via KDR are increasingly becoming understood. A key role for PKC has been identified in mediating VEGF-induced ERK activation and proliferation [13,23]. VEGF-dependent ERK activation mediates cytosolic phospholipase $A_2$ activation, causing the subsequent mobilization of arachidonic acid and, together with intracellular $Ca^{2+}$, increases the release of $PGI_2$ [12,13]. c-Src has been shown to associate with KDR and has also been implicated in mediating VEGF-induced NO and $PGI_2$ production [24]. Akt activation is an important mediator of the anti-apoptotic effect of VEGF and also induces the phosphorylation and activation of eNO synthase [20,25]. VEGF-stimulated FAK tyrosine phosphorylation in endothelial cells is associated with increased formation of stress fibres, recruitment of FAK to new focal adhesions and increased endothelial cell migration [22,26]; however, the functions of this pathway in VEGF endothelial signalling are not fully understood and the mechanism underlying VEGF-induced FAK phosphorylation is unknown.

FAK is an important component of the focal adhesion signalling complex [27,28] and is implicated in several fundamental cellular biological functions including migration, adhesion, survival, embryonic development and cell-cycle control [28–34]. A 159-residue region (the focal adhesion targeting domain, or FAT) in the C-terminal non-catalytic domain of FAK is essential for targeting to focal adhesions [35] and mediates

Abbreviations used: DAPI, 4,6-diamidino-2-phenylindole; ERK, extracellular signal-regulated protein kinase; FAK, focal adhesion kinase; FBS, foetal-bovine serum; HUVECs, human umbilical-vein endothelial cells; $PGI_2$, prostaglandin $I_2$; PI 3-kinase, phosphoinositide 3-kinase; PKC, protein kinase C; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; PP3, 4-amino-7-phenylpyrazol[3,4-d]pyrimidine; SH, Src homology; VEGF, vascular endothelial growth factor.

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its association with paxillin, which co-localizes to focal contacts and is a potential substrate for FAK [36,37]. Tyrosine phosphorylation of FAK is increased through the activation of Src [38,39] and has been identified as a common signalling pathway stimulated by diverse extracellular stimuli including several growth factors for receptor protein tyrosine kinases [22,40,41]. Specific phosphorylated tyrosine residues in FAK are thought to be critical for signal relay by mediating complex formation between FAK and other signalling molecules. Tyr-397 (Y397) is the major FAK autophosphorylation site and phosphorylation at this residue creates a high-affinity binding site for the Src homology (SH)2 domains of $pp60^{c\text{-}src}$ and $pp59^{c\text{-}fyn}$ [42]. FAK is also phosphorylated *in vitro* by Src at tyrosine residues 407, 576, 577, 861 and 925 [38,39]. Phosphorylation at Y576 and Y577 enhances FAK kinase activity and Y925 is a binding site for the SH2 domain protein GRB-2 [43] but the function(s) of other sites of tyrosine phosphorylation are currently unclear. Recent findings have also identified serine phosphorylation sites in FAK at Ser-722 (S722) and S910 that are regulated during mitosis [44]. Proline-rich motifs in the C-terminal domain mediate the association of FAK with the SH3-domain proteins including p130 Crk-associated substrate ($p130^{Cas}$) [45]. FAK also associates with other proteins, including the p85$\alpha$ subunit of PI 3-kinase [46] and talin [47]. The non-catalytic N-terminal region of FAK can associate with peptides corresponding to the cytoplasmic domains of integrins [48] but little else is known about the function of this domain.

Here we identify Y861 and Y397 as major sites of FAK phosphorylation in endothelial cells and demonstrate that VEGF induces selective FAK phosphorylation at Y861 and promotes the recruitment of phospho-Y861 FAK to new focal adhesions in endothelial cells. The Src inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) specifically blocks Y861 phosphorylation and recruitment of this phosphorylated form to focal adhesions. We show further that PP2 markedly inhibits VEGF-induced cell migration and VEGF-induced anti-apoptosis. These findings define Src-dependent FAK phosphorylation at Y861 as a novel VEGF-induced signal transduction pathway and suggest a role for this pathway in VEGF-induced chemotactic and anti-apopotic signalling.

## EXPERIMENTAL

### Cell culture

Human umbilical cords were obtained from full-term normal deliveries (maternity unit, University College Hospital, London, U.K.) and placed in Hanks balanced salt solution (Sigma) containing 1.7 mg/ml sodium bicarbonate, 12.5 mM Hepes and 100 $\mu$g/ml gentamicin sulphate. Endothelial cells were dispersed by incubation with 0.5 mg/ml collagenase for 10 min at 37 °C, collected by centrifugation at 200 ***g*** for 5 min and and left to attach overnight on 1% (w/v) gelatin in M199 medium supplemented with 20% (v/v) foetal-bovine serum (FBS) and 5 mM glutamine. Cells were subsequently cultured in endothelial basal medium (EBM), supplemented with 50 $\mu$g/ml gentamicin sulphate, 50 $\mu$g/ml Amphotericin B, 10 ng/ml human epidermal growth factor and 12 $\mu$g/ml bovine brain extract (all from Clonetics). For experimental purposes, cells were plated on 90 mm dishes or coverslips and left to grow to confluence before treatments.

### Western blotting

Equal quantities of protein were separated by SDS/PAGE and transferred to PVDF membranes (Millipore). The membranes were blocked with PBS/5% (v/v) non-fat milk for 30 min with agitation at 21 °C, then incubated for 1 h at 37 °C with primary antibody (2 $\mu$g/ml) diluted in blocking buffer. Membranes were washed in PBS/0.1% (v/v) Tween 20 and then incubated for 1 h with horseradish-peroxidase-conjugated secondary antibody at 37 °C. Immunoreactive bands were detected by chemiluminescence with ECL® reagents (Amersham). Bands were quantified by scanning densitometry and calculation of the density of individual bands with QuantiScan software.

### Immunoprecipitation

Confluent human umbilical-vein endothelial cells (HUVECs) were washed twice with serum-free EBM, treated with VEGF as indicated in the Figure legends and lysed in a buffer containing 50 mM Hepes, pH 7.4, 1% (v/v) Triton X-100, 0.5% (v/v) Nonidet P40, 150 mM NaCl, 10% (v/v) glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM disodium pyrophosphate, 100 mM NaF, 200 $\mu$M 4-(2-aminoethyl)benzenesulphonyl fluoride and 1 mM $Na_3VO_4$. Cells were lysed for 15 min at 4 °C, then centrifuged at 14000 ***g*** for 20 min. The lysates were transferred to fresh tubes containing anti-KDR antibody (2 $\mu$g/ml) (Santa Cruz) and incubated at 4 °C for 4 h. Immunoprecipitates were washed three times in lysis buffer, extracted in 2 × SDS/PAGE sample buffer and resolved by SDS/PAGE. Gels were transferred to Immobilon™ and membranes were blotted with antibody against phosphotyrosine (4G10; Upstate Biotechnology).

### Chemotaxis assay

Cell migration was measured in a modified Boyden chemotaxis chamber (NeuroProbe, Cabin John, MD, U.S.A.) as described previously [22]. M199 supplemented with 1% (w/v) BSA and VEGF, as indicated in the Figure legends, was placed in the bottom wells of the chamber. Polycarbonate filters with 8 $\mu$m pores (Osmonics Poretics) were preincubated in a 0.1% solution of collagen type I and placed between the bottom and top chambers. Cells were trypsinized and washed, then resuspended in M199 supplemented with 1% (w/v) BSA to give a final cell concentration of $3 \times 10^5$/ml. Cells (15000), with or without further additions as indicated, were placed into each well of the top chamber; the chemotaxis chambers were incubated at 37 °C for 6 h. After the incubation, unmigrated cells were removed from the top side of the filters, and migrated cells were stained with Pro-Diff (Braidwood Laboratories, Beckenham, Kent, U.K.). The stained cells were counted at × 100 magnification in four fields per well with the help of an eyepiece indexed graticule.

### Wound-healing assay

HUVECs cultured in 24-well plates were grown to confluence, washed with serum-free medium and wounded with a 200 $\mu$l pipette tip. Wounded monolayers were then incubated in serum-free medium containing additions as described, for 8 and 24 h. Cells were then fixed and stained with Pro-Diff; stained cells were photographed through a Zeiss Axiovert 25 microscope fitted with a Yashica 109 camera and Ilford FP4 film.

### Measurement of apoptosis

Subconfluent HUVECs in six-well plates were washed twice with serum-free medium and incubated for 24 h with the indicated additions. The cells were then trypsinized, collected by centrifugation and stained with fluorescein-conjugated annexin V and propidium iodide, using a kit in accordance with the manu-

facturer's instructions (Boehringer Mannheim). After staining, the cells were sorted by flow cytometry with a FACScan (Becton Dickinson). Annexin V-positive staining cells were counted as apoptotic cells. Cells that were propidium iodide-positive but annexin V-negative were not counted as apoptotic cells.

### Cell proliferation

HUVECs were seeded at a density of 4000 cells per well in 96-well plates. At 2 h after plating, medium was replaced with fresh medium containing 0.5% FBS and other additions as indicated, including $VEGF_{165}$ at 10 ng/ml. After incubation for 4 days, cells were stained with Pro-Diff (Braidwood Laboratories) and cell numbers were determined by microscopy in 12 fields per well with an eyepiece indexed graticule at $\times 100$ magnification.

### Immunofluorescent staining and microscopy

Confluent cultures of HUVECs on glass coverslips were fixed and permeabilized by immersion in precooled methanol for 10 min at $-20$ °C and then washed in PBS. Cells were incubated in blocking buffer with 2% FBS for 30 min, rinsed three times in PBS and then incubated in primary antibody (1–10 $\mu$g/ml). The cells were washed three more times before incubation for 30 min with FITC-conjugated secondary antibodies or 4,6-diamidino-2-phenylindole (DAPI) diluted in PBS/2% (w/v) FBS. The coverslips were inverted on microscope slides, mounted in Vectashield (Vector Labs) and sealed. Cells were studied and photographed on a Zeiss Axiovert 100M microscope with a $63\times$ oil-immersion objective lens (numerical aperture 1.4) linked to a Yamamatsu DCC camera and Open Lab Improvision software.

### Materials

PP2, 4-amino-7-phenylpyrazol[3,4-d]pyrimidine (PP3) and LY294002 were from Calbiochem. Monoclonal antibodies against FAK came from Transduction Laboratories. Polyclonal antibodies against FAK C-terminal residues 1033–1052 (FAK-C), and FAK N-terminal residues 2–18 (FAK-N) were from Santa Cruz. Phospho-specific antibodies against FAK phosphorylated at Y397, Y407, Y576, Y577, Y861 and Y925 were from Biosource International. Horseradish-peroxidase-conjugated secondary antibodies against mouse and rabbit immunoglobulins were from Amersham International. Fluorochrome-conjugated secondary antibodies were from Dako. All other reagents were of the highest grade available.

## RESULTS

Western blotting of whole-cell extracts prepared from unstimulated HUVECs with antibodies against FAK phosphorylated at residues Y397, Y407, Y861, Y925, Y576 and Y577 showed that these cells contained significant immunoreactivity to $p125^{FAK}$ phosphorylated at Y397 and pY861, a smaller amount of immunoreactivity to $p125^{FAK}$ phosphorylated at Y407 and no detectable immunoreactivity to other phosphorylated FAK residues (results not shown). We demonstrated previously that VEGF stimulated FAK tyrosine phosphorylation and promoted FAK association with new focal adhesions in HUVECs [22]. As shown in Figure 1, VEGF caused a marked increase in the immunostaining of focal adhesions with antibodies against FAK phosphorylated at Y861 and Y397. After 10 min there was an increase in the number of stained focal adhesions and of a more punctate staining characteristic of focal complexes. After 60 min of treatment with VEGF, there was a marked increase in both the number and the size of focal adhesions decorated with antibodies against FAK phosphorylated at Y861 and Y397. Focal adhesion staining declined after 120 min but remained above that seen in control untreated cells.

To investigate whether increased immunofluorescent localization of phosphorylated FAK to focal adhesions was the result of increased phosphorylation as opposed to recruitment of phosphorylated FAK to newly formed focal adhesions, whole cell extracts prepared from VEGF-treated cells were immunoblotted with antibodies against FAK phosphorylated at Y861 and Y397. The results showed that VEGF caused a marked increase in FAK tyrosine phosphorylation at Y861, which was evident after 10 min and was sustained above the control level 4 and 24 h after the addition of VEGF (Figure 2A). It was observed that, in some experiments, phosphorylation at Y861 was increased at earlier times (5 and 10 min) but subsequently declined to near the basal level after 60 min (Figure 2C). However, a decrease in Y861 phosphorylation at 60 min was not observed in most experiments (compare with Figure 3A). Quantification of the results from several independent experiments showed that VEGF increased Y861 phosphorylation above the control unstimulated level by $3.0\pm0.45$-fold ($n=5$), $2.3\pm0.65$-fold ($n=3$) and $3.5\pm0.3$-fold ($n=4$) after 10 min, 60 min and 4 h respectively (Figure 2C). In all experiments, immunoblotting of parallel samples with an antibody that recognized the FAK N-terminal domain showed that levels of $p125^{FAK}$ did not change significantly after different times of treatment with VEGF (Figure 2). In contrast with its effect on Y861 phosphorylation, VEGF caused no significant increase in the total cellular level of FAK tyrosine phosphorylation at Y397 (Figure 2) or at other phosphorylation sites (results not shown).

Previous findings of an association between FAK and the platelet-derived growth factor $\beta$-receptor [49,50] prompted us to determine whether VEGF could stimulate FAK Y861 phosphorylation by direct association between KDR and FAK. However, FAK Western blotting of KDR immunoprecipitates did not reveal detectable co-immunoprecipitation of these molecules (results not shown). Because Src is known to stimulate FAK Y861 phosphorylation [38,39], we next investigated whether VEGF-induced Y861 phosphorylation in HUVECs was mediated via Src. The role of Src in mediating VEGF-induced FAK phosphorylation at Y861 was examined with the pyrazolopyrimidine PP2, a selective inhibitor of Src family kinases [51] at concentrations (1–10 $\mu$M) previously shown not to inhibit FAK kinase activity [52]. VEGF-induced FAK phosphorylation at Y861 was abolished in the presence of the specific Src inhibitor PP2 (Figure 3), whereas the inactive PP2 analogue PP3 caused no decrease in VEGF-stimulated Y861 phosphorylation compared with PP2. PP2 had no detectable inhibitory effect on FAK phosphorylation at Y397; similarly to the results obtained in Western blots, PP2 markedly decreased VEGF-stimulated Y861 phosphorylation in focal adhesions but had little effect on immunofluorescent staining of focal adhesions with antibody against $pY397^{FAK}$ (Figure 3). PP2 also caused no significant loss of focal adhesion immunostaining obtained with an antibody against total FAK (results not shown).

The inhibitory effect of PP2 on VEGF-induced Y861 phosphorylation was not due to a non-specific effect on VEGF receptor activation or other downstream signalling pathways. Thus the VEGF-induced activation of either ERKs 1/2 or Akt was unaffected by PP2; in addition the Src inhibitor had no effect on the stimulation of KDR tyrosine phosphorylation by VEGF, as shown by anti-phosphotyrosine immunoblots of KDR immunoprecipitates (Figure 4).

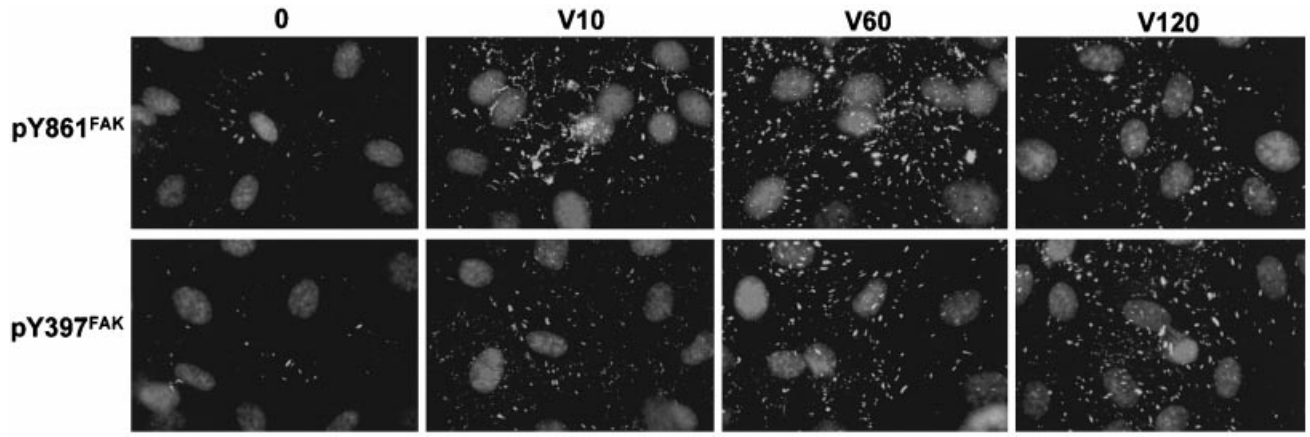


**Figure 1 VEGF induces focal adhesion association of FAK tyrosine phosphorylated at Y397 and Y861**

Confluent HUVECs were treated with VEGF for the durations indicated (in mins), fixed and permeabilized, then immunostained with antibodies against FAK phosphorylated at Y397 (pY397$^{FAK}$) or Y861 (pY861$^{FAK}$) and secondary antibodies conjugated with FITC. Cells were counterstained with DAPI to reveal nuclei. Focal adhesions appear as short linear patches of intense staining. The results shown are representative of three similar experiments. Magnification, $\times 63$.

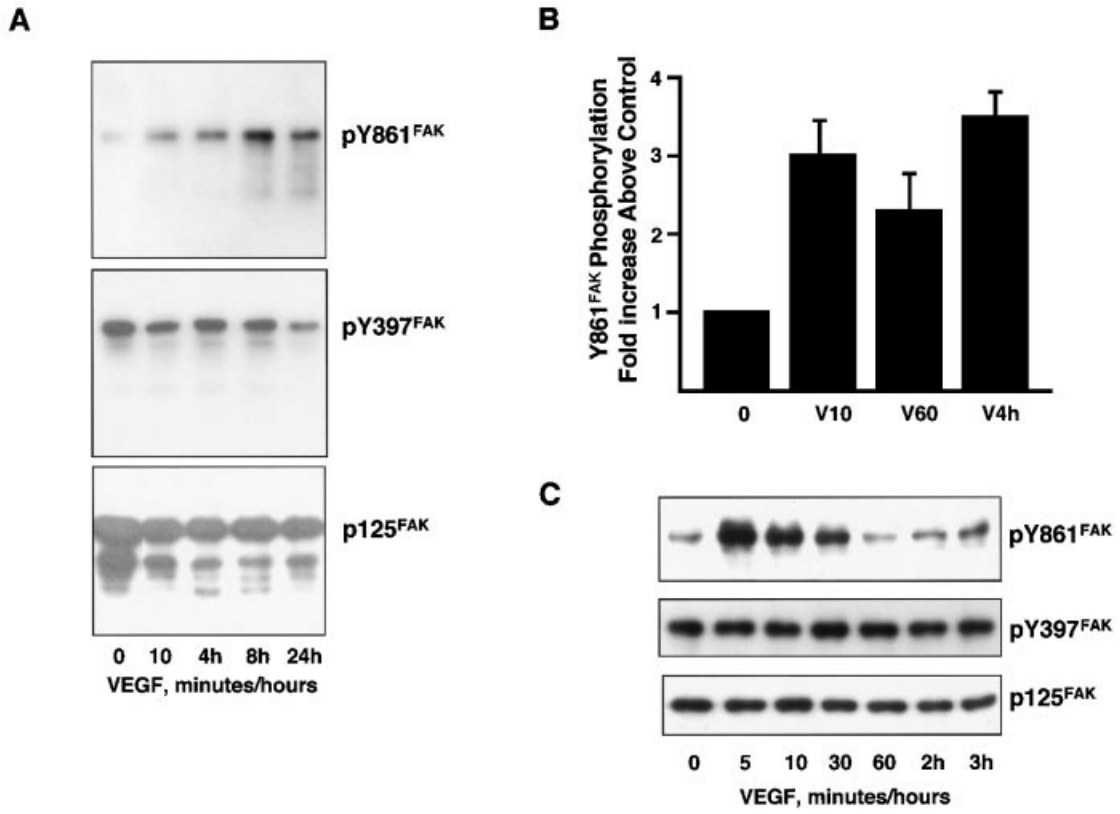


**Figure 2 VEGF selectively induces tyrosine phosphorylation of FAK at Y861**

(**A**) Confluent HUVECs were treated with VEGF for the durations indicated; cell extracts were prepared and then immunoblotted with antibodies against FAK phosphorylated at Y861 (pY861$^{FAK}$, top panel) or Y397 (pY397$^{FAK}$, middle panel) or antibody recognizing the N-terminal FAK domain (p125$^{FAK}$, bottom panel). The results shown are representative of five similar experiments. (**B**) Quantification of FAK phosphorylation at Y861 after VEGF treatment for 10 min, 60 min and 4 h was performed by scanning densitometry; the areas of the bands were integrated with QuantiScan software. Results are presented as fold increases (means $\pm$ S.E.M.; $n = 3$–5) above the control value. (**C**) Time course of FAK phosphorylation after treatment with 25 ng/ml VEGF for the indicated durations, showing transient phosphorylation at Y861. Other experimental details were as described for (**A**).

To examine whether Src-mediated VEGF-induced Y861 phosphorylation could have a role in endothelial cell migration induced by VEGF, the effect of PP2 on VEGF-stimulated chemotaxis was determined. VEGF induced a striking increase in the directed cell migration of HUVECs and incubation of parallel cultures of HUVECs in the presence of PP2 markedly decreased

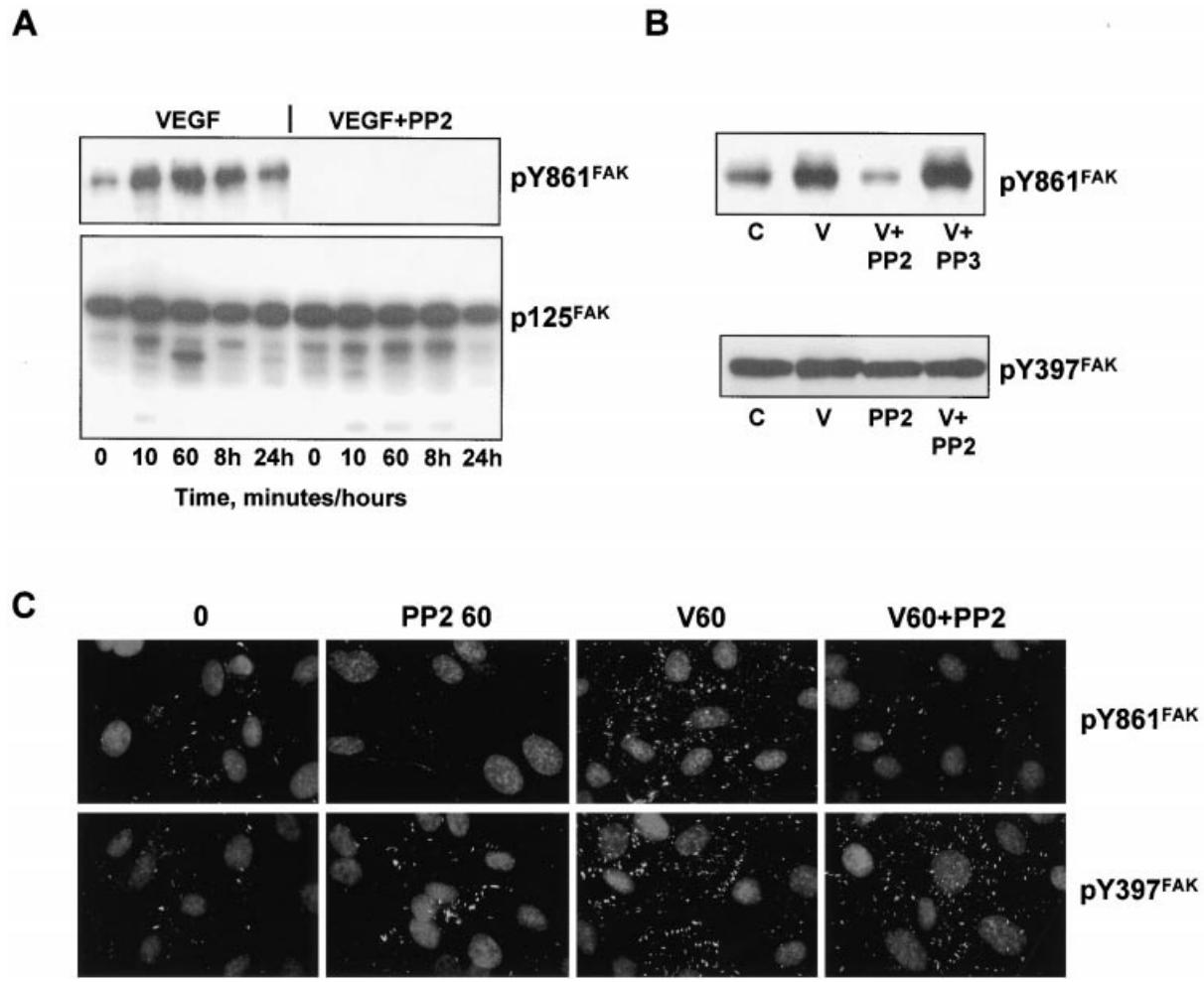


**Figure 3 VEGF activates Src and VEGF-induced FAK Y861 phosphorylation is inhibited by PP2 independently of phosphorylation at Y397**

(**A**, **B**) Confluent HUVECs were treated as indicated for 60 min in the presence or absence of either PP2 (3 $\mu$M) or PP3 (3 $\mu$M) and subsequently incubated with 25 ng/ml VEGF (VEGF, V) for the durations indicated (**A**) or for 10 min (**B**). Whole cell extracts were then prepared and immunoblotted with antibodies against FAK phosphorylated at Y861 (pY861$^{FAK}$), Y397 (pY397$^{FAK}$) or antibody recognizing the N-terminal FAK domain (p125$^{FAK}$). The results shown are representative of at least three similar experiments. (**C**) HUVECs were treated as indicated for 60 min in the presence or absence of PP2 (3 $\mu$M) and subsequently incubated for a further 60 min with 25 ng/ml VEGF. Cells were then fixed and permeabilized and immunostained with antibody against FAK phosphorylated at Y861 (pY861$^{FAK}$) or Y397 (pY397$^{FAK}$). Nuclei were made visible by staining with DAPI. The results shown are representative of two similar experiments.

the chemotactic response to VEGF (Figure 5A). The effect of Src inhibition on VEGF-induced cell migration was also examined in a wound-healing assay. After the wounding of HUVEC monolayers with a pipette tip, incubation with VEGF stimulated the migration of cells into the wounded area. VEGF-induced migratory activity was visible 8 h after wounding; extensive repopulation of the wounded area had occurred after 24 h (Figure 5B). In the presence of PP2, VEGF-stimulated migration of cells from the wound margin was limited after 8 h; although some cell migration into the wounded area had occurred after 24 h, the extent of repopulation was markedly less than with VEGF alone (Figure 5B).

Given that VEGF is an endothelial survival factor and that FAK has been strongly implicated in the survival signalling relayed through focal adhesions from integrin–matrix interactions, we next investigated whether Src and Src-mediated Y861 phosphorylation could have a role in VEGF-dependent anti-apoptosis. Consistent with the findings of other investigators was our observation that VEGF caused a marked decrease in the apoptosis of HUVECs induced by serum deprivation, as indicated by a VEGF-dependent decrease in cell-surface annexin V binding. PP2 alone caused a modest increase in the basal level of apoptosis in control serum-deprived HUVECs and abolished the anti-apoptotic effect of VEGF (Figure 6A). PP3 had little effect on the anti-apoptotic effect of VEGF (results not shown). Furthermore, treatment with PP2 diminished the increase in cell proliferation in response to VEGF (Figure 6B).

We next examined whether the ability of PP2 to inhibit the anti-apoptotic effect of VEGF was associated with increased FAK cleavage. Endothelial cell apoptosis induced by serum deprivation was characterized by increased cell detachment and FAK proteolysis to N-terminal fragments [53–55], and detached apoptotic cells contained no or a greatly decreased level of p125$^{FAK}$ (Figure 7A). The anti-apoptotic effect of VEGF was associated with a decrease in FAK proteolysis to an N-terminal 80 kDa product in detached cells compared with the effect of

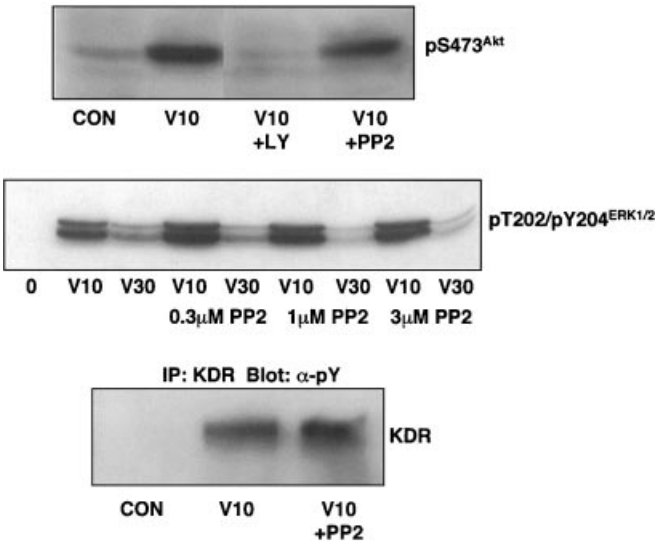


**Figure 4 Inhibition of VEGF-induced Y861 FAK phosphorylation by PP2 is not due to inhibition of ERK, Akt or KDR**

HUVECs were treated as indicated for 60 min in the presence or absence of PP2 (3 $\mu$M) and subsequently incubated with 25 ng/ml VEGF for the durations indicated (in mins). Whole cell extracts were then prepared and immunoblotted with antibodies against Akt phosphorylated at S473 (top panel) or ERKs1/2 phosphorylated at T202 and Y204 (middle panel). In some experiments KDR immunoprecipitates were prepared and immunoblotted with anti-phosphotyrosine antibody (4G10, bottom panel).

serum deprivation alone (Figure 7A). However, PP2 did not sigificantly alter p125$^{FAK}$ expression in adherent cells after 24 h (Figures 3A and 7A). The inhibitor also did not increase FAK proteolysis to an 80 kDa band in detached cells and did not reverse the inhibitory effect of VEGF on FAK proteolysis (Figure 7A).

As the activation of Akt dependent on PI 3-kinase has been identified as a major pathway mediating VEGF-induced cell survival, we compared the effects of PP2 on FAK and VEGF-dependent anti-apoptosis with those of the PI 3-kinase inhibitor LY294002. LY294002 caused a marked decrease in p125$^{FAK}$ expression in adherent cells after 24 h and a concomitant increase in FAK proteolysis to N-terminal 80 and 35 kDa cleavage products in detached cells from the same cultures (Figure 7A). In contrast with the effect of PP2, treatment with LY294002 for 1 h had a much weaker effect than PP2 on VEGF-stimulated FAK phosphorylation at Y861 (Figure 7B). LY294002 caused a modest decrease in Y397 phosphorylation. Treatment with 10 $\mu$M LY294002 alone for 24 h produced a marked increase in HUVEC apoptosis above that induced by serum withdrawal alone and completely inhibited the anti-apoptotic effect of VEGF (Figure 7C). At the same concentration, LY294002 blocked VEGF-induced Akt activation and abolished basal Akt activity (results not shown).

## DISCUSSION

Although Y397 is the major autophosphorylation site in FAK and has a well-established role in mediating FAK associations with pp60$^{c\text{-}src}$ and pp59$^{c\text{-}fyn}$, the role of phosphorylation at Y861 is unclear. Y861 and Y397 were the major FAK tyrosine phosphorylation sites in resting endothelial cells and the findings presented here demonstrate that VEGF selectively increases Y861 phosphorylation without affecting phosphorylation at Y397. Consistent with our previous finding that VEGF promotes association of FAK with newly formed focal adhesions in HUVECs [22] was our observation that VEGF also increased

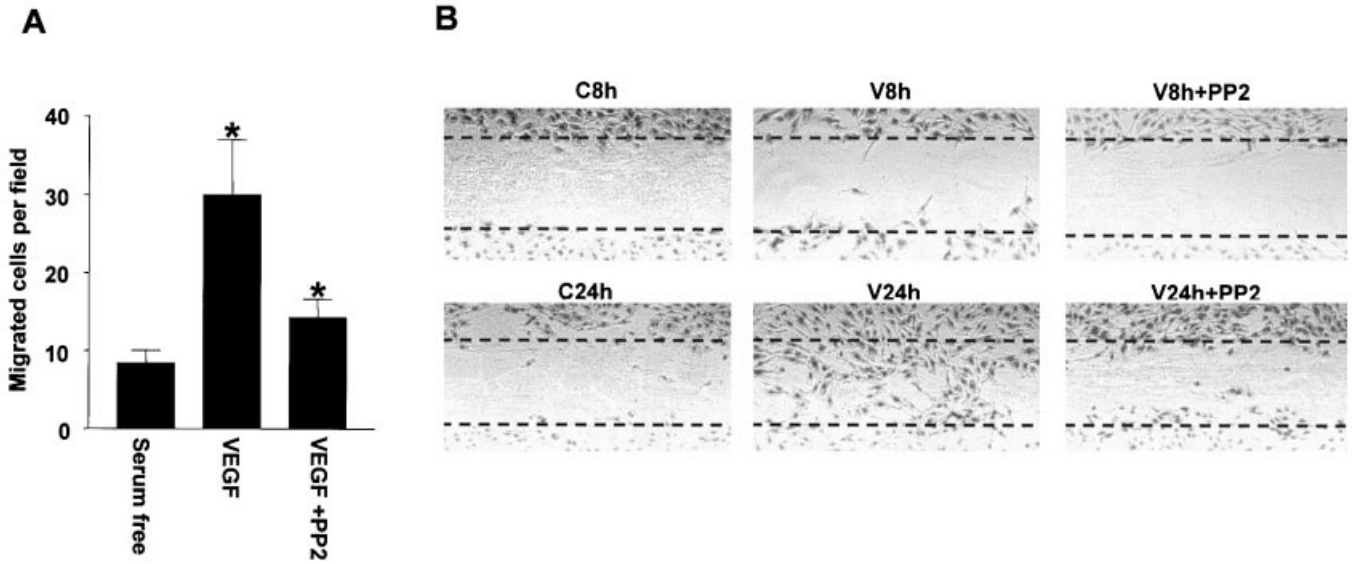


**Figure 5 PP2 inhibits VEGF-induced chemotaxis and wound-healing migration**

(**A**) M199 medium in the absence (Serum free) or presence of 25 ng/ml VEGF was placed in the bottom wells of a 48-well chemotaxis chamber. Cells (15000) in the absence or presence of 3 $\mu$M PP2 were placed in the top wells of the chamber. After 6 h, migrated cells were stained and counted. Results are means $\pm$ S.E.M. for migrated cells counted from quadruplicate determinations. Similar results were obtained from three independent experiments. $^*P < 0.03$ for VEGF compared with VEGF + PP2. (**B**) HUVEC monolayers were wounded with a pipette tip and then incubated in serum-free medium in the absence (C, control) or presence (V, VEGF) of 25 ng/ml VEGF, or in the presence or absence of 25 ng/ml VEGF plus 3 $\mu$M PP2. After 8 or 24 h, cells were fixed and stained and then photographed. The wound margins are indicated by broken lines. Representative fields are shown from experiments performed in quadruplicate.

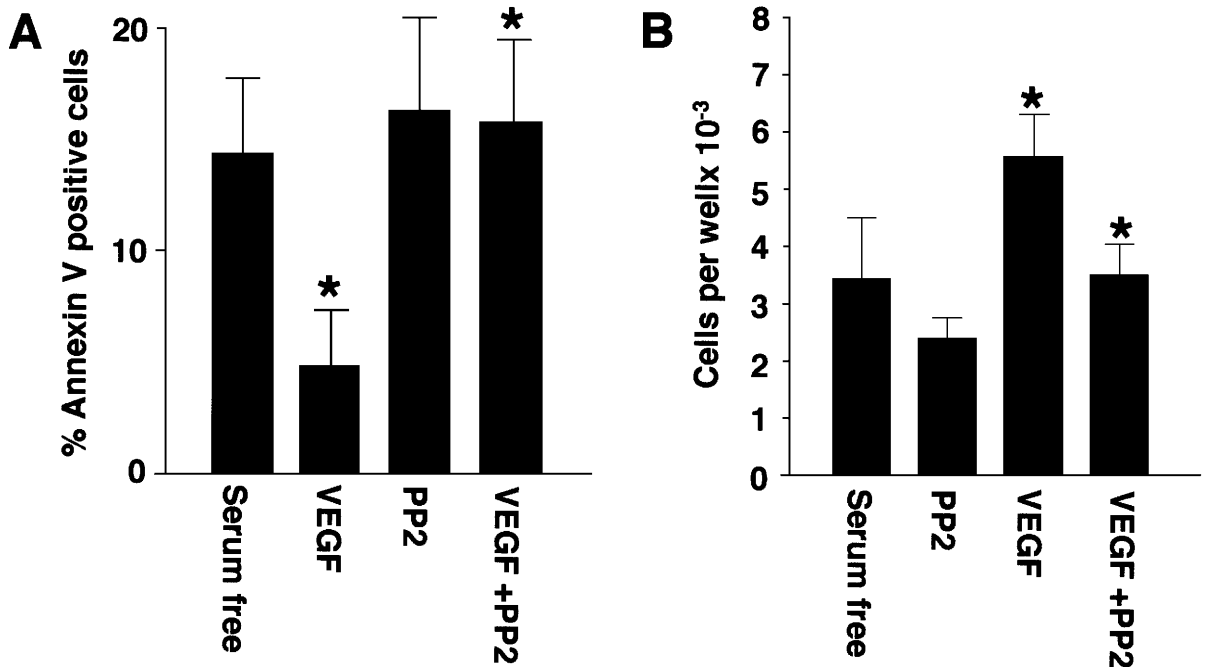


**Figure 6 Inhibition of VEGF-mediated anti-apoptosis (A) and cell proliferation (B) by PP2**

(**A**) Subconfluent HUVECs were incubated for 48 h with either serum-free medium with or without 25 ng/ml $VEGF_{165}$, or with 3 $\mu$M PP2 in the presence or absence of 25 ng/ml $VEGF_{165}$. The frequency of apoptotic cells was determined by flow-cytometric analysis of annexin V staining as described in the Experimental section. Results are means $\pm$ S.E.M. for apoptotic cells calculated from the results of three independent experiments each performed in duplicate.*$P < 0.01$ for VEGF versus VEGF + PP2. (**B**) Proliferation of HUVECs stimulated by the addition of 10 ng/ml of VEGF was determined in the absence or presence of the peptides at the indicated concentrations. Resuts are means $\pm$ S.E.M. of cell proliferation stimulated by VEGF calculated from triplicate determinations. Similar results were obtained from three independent experiments. *$P < 0.05$ for VEGF versus VEGF + PP2.

focal adhesion localization of FAK phosphorylated at both Y861 and Y397. In the case of phospho-Y397 FAK, this presumably reflects increased recruitment of total FAK to focal adhesions, whereas we conclude that increased focal adhesion association of phospho-Y861 FAK is a result of phosphorylation of FAK at this site either before or after focal adhesion targeting.

In a minority of experiments it was observed that the stimulation of Y861 phosphorylation by VEGF was strikingly transient. This variation in the kinetics of stimulated Y861 phosphorylation agrees with our previous work, which showed similar differences in the time dependence for total VEGF-induced FAK tyrosine phosphorylation in HUVECs [22]. Such variations might in part reflect heterogeneity in the responses of different populations of cells. The decline in VEGF-induced Y861 phosphorylation after 60 min in some experiments could additionally be a consequence of VEGF receptor down-regulation in a particular cell preparation, whereas increased FAK Y861 phosphorylation after 60 min in some experiments and after longer durations in other experiments might result from longer-term effects of VEGF on cell survival and/or actin cytoskeletal and focal adhesion organization.

We previously demonstrated that VEGF induces FAK tyrosine phosphorylation but the mechanism involved was not established [22]. A salient finding of the present paper is that VEGF-induced Y861 phosphorylation is blocked by the specific Src family kinase inhibitor PP2. The effects of PP2 were not the result of a non-specific decrease in KDR activation or other signal transduction pathways distal to KDR because the inhibitor had no effect on VEGF-stimulated KDR phoshorylation, ERK activation or Akt activation. Our findings are consistent with previous studies showing that Src induces FAK Y861 phosphorylation *in vitro* and *in vivo* [38,39] and that PP2 inhibits agonist-induced FAK phosphorylation at Y577 in Swiss 3T3 cells [52]. The present results show that Src mediates phosphorylation specifically at Y861, independently of a significant effect on Y397 or other sites. Given that Y397 has been identified as the major binding site for Src, our findings suggest that Y861 phosphorylation is stimulated independently of an association between Src and FAK. However, our preliminary data show that VEGF increases an association between $pp60^{c\text{-}src}$ and FAK (I. Zachary and J. Kabir, unpublished work), suggesting that VEGF-stimulated phosphorylation at Y861 might enhance the ability of FAK to engage in complex formation with Src.

Because FAK and Src are strongly implicated in signal transduction linked to cell migration, the marked inhibitory effect of PP2 on VEGF-induced chemotaxis and wound-healing cell migration is consistent with a role for Src-mediated FAK Y861 phosphorylation in the migratory response to VEGF, although it does not in itself provide direct evidence for such a role. The results of the wound-healing assay indicate that PP2 does not completely block migratory activity but instead retards cell movement and repopulation of the wounded area. Moreover, because Src inhibition had little effect on focal adhesion localization of FAK phosphorylated at Y397 and did not cause a rapid marked alteration in cell morphology or the integrity of focal adhesions, it seems that Y861 dephosphorylation alone is insufficient to promote the rapid disengagement of FAK from focal adhesions or focal adhesion disassembly. A decrease in the rate of focal adhesion turnover and/or a disruption of chemotactic signalling through focal adhesions mediated directly by or distal to FAK Y861 phosphorylation are potential mechanisms

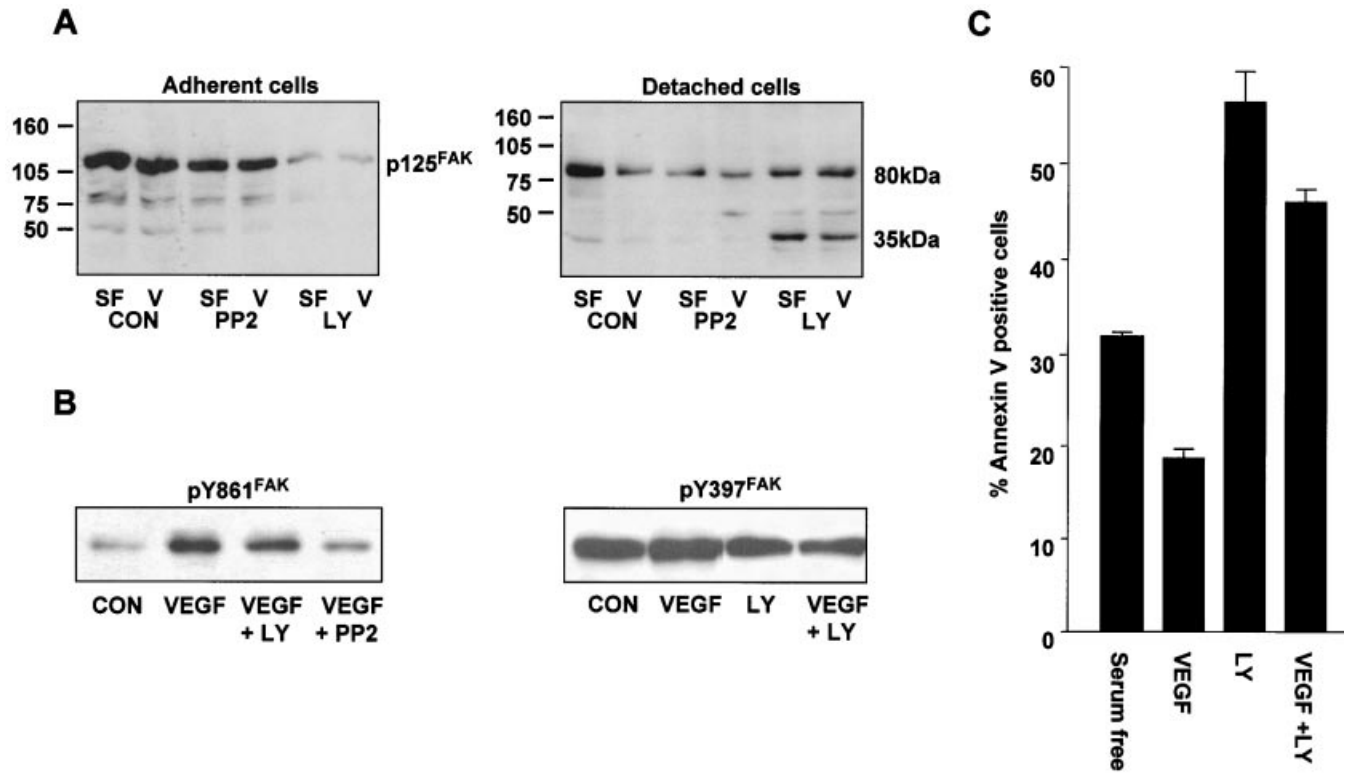


**Figure 7 Inhibition of VEGF-mediated anti-apoptosis by PP2 is independent of increased FAK proteolysis: comparison with effect of LY294002**

(**A**) Cells were incubated for 24 h in serum-free medium in the absence (SF) or presence (V) of 25 ng/ml VEGF and in the presence or absence of either 3 $\mu$M PP2 or 10 $\mu$M LY294002 (LY). At the end of incubations the culture medium containing floating detached cells was removed and cells were collected by centrifugation in a Microfuge at top speed for 15 min. Whole cell extracts were prepared from adherent cells and from the floating cell fraction collected from the medium, and were immunblotted with antibody recognizing the N-terminal FAK domain ($p125^{FAK}$). The positions of molecular mass markers (in kDa) are indicated at the left of the autoradiograms; those of $p125^{FAK}$ and 80 and 35 kDa N-terminal cleavage products are indicated at the right. The results shown are representative of three similar experiments. (**B**) Cells were treated for 10 min with (VEGF) or without (CON) 25 ng/ml VEGF in the presence or absence of either 3 $\mu$M PP2 or 10 $\mu$M LY294002 (LY). Whole cell extracts were prepared and blotted with antibody against FAK phosphorylated at either Y861 ($pY861^{FAK}$) or Y397 ($pY397^{FAK}$) (**C**) Subconfluent HUVECs were incubated with serum-free medium with or without 25 ng/ml $VEGF_{165}$, and in the presence or absence of 10 $\mu$M LY294002 (LY) for 24 h. The frequency of apoptotic cells was determined by flow cytometric analysis of annexin V staining. Results are frequencies (means $\pm$ S.E.M.) of apoptotic cells calculated from the results of three independent experiments, each performed in duplicate.

underlying the inhibition of VEGF-induced cell migration by PP2; further investigation of these possibilities is warranted.

An important function of VEGF in which FAK has a potential role as a signal transducer is the promotion of cell survival and activation of anti-apoptotic signalling pathways. Integrin-dependent FAK signalling through focal adhesions is also thought to be critical for maintaining survival signals in endothelial and other adherent cells [56]. It can be speculated that the significant level of basal Y861 phosphorylation detected in HUVECs might be linked to the relaying of constitutive cell-survival signals. Our preliminary data indicate that the adhesion of HUVECs to the extracellular matrix components fibronectin, collagen and vitronectin induces a marked increase in phosphorylation at Y861 (C. Ham and I. Zachary, unpublished work) suggesting that basal phosphorylation at this site is maintained through integrin engagment. The finding that PP2 inhibited the anti-apoptotic effect of VEGF suggests a role for Src and Src-dependent phosphorylation at Y861 in VEGF survival signalling. However, our results suggest that inhibition of Src by itself might not be sufficient to elicit a complete apoptotic response. Thus, whereas PP2 increased the surface binding of annexin V, which is a marker of early apoptosis, the fact that this compound did not prevent the VEGF-mediated inhibition of FAK proteolysis and did not itself cause any increase in FAK proteolysis indicates that Src inhibition and loss of Y861 phosphorylation alone do not significantly increase later apoptotic cell detachment leading to FAK cleavage. Such a conclusion would also be in agreement with the retention of phospho-Y397 FAK in focal adhesions observed after treatment with PP2.

Comparison of the effects of Src inhibition with those of the specific PI 3-kinase inhibitor LY294002 on FAK Y861 phosphorylation and apoptosis showed that, in contrast with PP2, LY294002 had a pronounced apoptotic effect acting alone and also markedly decreased $p125^{FAK}$ expression in adherent cells and increased FAK proteolysis in detached cells. LY294002 caused only a partial reduction in VEGF-induced FAK Y861 phosphorylation, indicating that PI 3-kinase does not make a major contribution to this pathway. Taken together with the fact that PP2 does not affect VEGF-induced Akt activation, these findings indicate that VEGF signals independently through the Src/FAK and PI 3-kinase/Akt pathway. Furthermore, our results indicate that, whereas a major effect of PP2 is inhibition of FAK Y861 phosphorylation, decreased $p125^{FAK}$ expression and increased FAK cleavage are likely to be important in apoptosis mediated through the inhibition of PI 3-kinase by LY294002. Indeed, the finding that PI 3-kinase inhibition was markedly apoptotic and induced FAK cleavage in the absence of VEGF strongly suggests that the maintenance of basal PI

3-kinase/Akt activity is an important survival mechanism in HUVECs. It is unclear whether basal Akt activity is required for the maintenance of normal levels of FAK expression or whether the decrease in FAK expression is a secondary consequence of apoptosis induced by suppression of PI 3-kinase/Akt activity. These results suggest that inhibition of the Src/FAK and PI 3-kinase/Akt pathways has distinct consequences for cell survival: in the case of Src/FAK, inhibition leads to FAK dephosphorylation and features of early apoptosis, whereas PI 3-kinase/Akt inhibition causes FAK instability and full-blown programmed cell death.

Although we do not exclude the possibility that the inhibition of Src might have other effects independently of the inhibition of FAK Y861 phosphorylation, the fact that FAK is both a major target for Src and is strongly implicated in the mechanisms underlying cell migration and survival supports the argument that VEGF-induced FAK phosphorylation mediated via Src contributes to the promotion of both chemotactic and anti-apoptotic responses to VEGF. These findings acquire added importance in the light of recent work demonstrating a selective requirement for pp60$^{c\text{-}src}$ during VEGF-induced angiogenesis and permeability *in vivo* [57]. Elucidation of the connections between the Src-dependent Y861 phosphorylation pathway and downstream and proximal molecules are likely to yield novel insights into the signal transduction mechanisms mediating the biological actions of VEGF.

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## REFERENCES


1 Ferrara, N. (1996) Vascular endothelial growth factor. Eur. J. Cancer **32A**, 2413–2422
2 Risau, W. (1997) Mechanisms of angiogenesis. Nature (London) **386**, 671–674
3 Zachary, I., Mathur, A., Yla-Herttuala, S. and Martin, J. F. (2000) Vascular protection: a novel, non-angiogenic cardiovascular role for vascular endothelial growth factor. Arterioscler. Thromb. Vasc. Biol. **20**, 1512–1520
4 Stavri, G., Hong, Y., Zachary, I., Breier, G., Baskerville, P., Yla-Herttualla, S., Risau, W., Martin, J. and Erusalimsky, J. (1995) Hypoxia and platelet-derived growth factor-BB synergistically upregulate the expression of vascular endothelial growth factor in vascular smooth muscle cells. FEBS Lett. **358**, 311–315
5 Stavri, G., Zachary, I., Baskerville, P., Martin, J. and Erusalimsky, J. (1995) Basic fibroblast growth factor upregulates the expression of vascular endothelial growth factor in vascular smooth muscle cells. Circulation **92**, 5–8
6 Pertovaara, L., Kaipainen, A., Mustonen, T., Orpana, A., Ferrara, N., Saksela, O. and Alitalo, K. (1994) Vascular endothelial growth factor is induced in response to transforming growth factor-$\beta$ in fibroblastic and epithelial cells. J. Biol. Chem. **269**, 6271–6274
7 Gospodarowicz, D., Abraham, J. A. and Schilling, J. (1989) Isolation and characterization of a vascular endothelial cell mitogen produced by pituitary-derived folliculo stellate cells. Proc. Natl. Acad. Sci. U.S.A. **86**, 7311–7315
8 Waltenberger, J., Claesson-Welsh, L., Siegbahn, A., Shibuya, M. and Heldin, C. H. (1994) Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. J. Biol. Chem. **269**, 26988–26995
9 Alon, T., Hemo, I., Itin, A., Pe'er, J., Stone, J. and Keshet, E. (1995) Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. Nat. Med. **1**, 1024–1028
10 Laitinen, M., Zachary, I., Breier, G., Pakkanen, T., Hakkinen, T., Luoma, J., Abedi, H., Risau, W., Soma, M., Laakso, M. et al. (1997) VEGF gene transfer reduces intimal thickening via increased production of nitric oxide in carotid arteries. Hum. Gene Ther. **8**, 1737–1744
11 van der Zee, R., Murohara, T., Luo, Z., Zollmann, F., Passeri, J., Lekutat, C. and Isner, J. M. (1997) Vascular endothelial growth factor/vascular permeability factor augments nitric oxide release from quiescent rabbit and human vascular endothelium. Circulation **95**, 1030–1037
12 Wheeler-Jones, C., Abu-Ghazaleh, R., Cospedal, R., Houliston, R. A., Martin, J. and Zachary, I. (1997) Vascular endothelial growth factor stimulates prostacyclin production and activation of cytosolic phospholipase A2 in endothelial cells via p42/p44 mitogen-activated protein kinase. FEBS Lett. **420**, 28–32
13 Gliki, G., Abu-Ghazaleh, R., Jezequel, S., Wheeler-Jones, C. and Zachary, I. (2001) Vascular endothelial growth factor-induced prostacyclin production is mediated by a protein kinase C (PKC)-dependent activation of extracellular signal-regulated protein kinases 1 and 2 involving PKC-$\delta$ and by mobilization of intracellular $Ca^{2+}$. Biochem. J. **353**, 503–512
14 Connolly, D. T., Olander, J. V., Heuvelman, D., Nelson, R., Monsell, R., Siegel, N., Haymore, B. L., Leimgruber, R. and Feder, J. (1989) Human vascular permeability factor. Isolation from U937 cells. J. Biol. Chem. **264**, 20017–20024
15 Neufeld, G., Cohen, T., Gengrinovitch, S. and Poltorak, Z. (1999) Vascular endothelial growth factor (VEGF) and its receptors. FASEB J. **13**, 9–22
16 Zachary, I. and Gliki, G. (2001) Signaling transduction mechanisms mediating biological actions of the vascular endothelial growth factor family. Cardiovasc. Res. **49**, 568–581
17 Gille, H., Kowalski, J., Yu, L., Chen, H., Pisabarro, M. T., Davis-Smyth, T. and Ferrara, N. (2000) A repressor sequence in the juxtamembrane domain of Flt-1 (VEGFR1) constitutively inhibits vascular endothelial growth factor-dependent phosphatidylinositol 3′-kinase activation and endothelial cell migration. EMBO J. **19**, 4064–4073
18 Soker, S., Takashima, H., Miao, G., Neufeld, G. and Klagsburn, M. (1998) Neuropilin-1 is expressed by enothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell **92**, 735–745
19 Xia, P., Aiello, L. P., Ishii, H., Jiang, Z. Y., Park, D. J., Robinson, G. S., Takagi, H., Newsome, W. P., Jirousek, M. R. and King, G. L. (1996) Characterization of vascular endothelial growth factor's effect on the activation of protein kinase C, its isoforms, and endothelial cell growth. J. Clin. Invest. **98**, 2018–2026
20 Gerber, H. P., McMurtrey, A., Kowalski, J., Yan, M., Keyt, B. A., Dixit, V. and Ferrara, N. (1998) Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. J. Biol. Chem. **273**, 30336–30343
21 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
22 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
23 Takahashi, T., Ueno, H. and Shibuya, M. (1999) VEGF activates protein kinase C-dependent, but Ras-independent Raf-MEK-MAP kinase pathway for DNA synthesis in primary endothelial cells. Oncogene **18**, 2221–2230
24 He, H., Venema, V. J., Gu, X., Venema, R. C., Marrero, M. B. and Caldwell, R. B. (1999) Vascular endothelial growth factor signals endothelial cell production of nitric oxide and prostacyclin through flk-1/KDR activation of c-Src. J. Biol. Chem. **274**, 25130–25135
25 Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R. and Zeiher, A. M. (1999) Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature (London) **399**, 601–605
26 Rousseau, S., Houle, F., Kotanides, H., Witte, L., Waltenberger, J., Landry, J. and Huot, J. (2000) Vascular endothelial growth factor (VEGF)-driven actin-based motility is mediated by VEGFR2 and requires concerted activation of stress-activated protein kinase 2 (SAPK2/p38) and geldanamycin-sensitive phosphorylation of focal adhesion kinase. J. Biol. Chem. **275**, 10661–10672
27 Zachary, I. and Rozengurt, E. (1992) Focal adhesion kinase (p125$^{FAK}$): a point of convergence in the action of neuropeptide, integrins and oncogenes. Cell **71**, 891–894
28 Hanks, S. K. and Polte, T. R. (1997) Signalling through focal adhesion kinase. Bioessays **19**, 137–145
29 Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M., Yamamoto, T. and Aizawa, S. (1995) Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. Nature (London) **377**, 539–544
30 Richardson, A. and Parsons, J. T. (1996) A mechanism for regulation of the adhesion-associated protein tyrosine kinase pp125$^{FAK}$. Nature (London) **380**, 538–540
31 Gilmore, A. P. and Romer, L. H. (1996) Inhibition of focal adhesion kinase (FAK) signalling in focal adhesions decreases cell motility and proliferation. Mol. Biol. Cell. **7**, 1209–1224
32 Frisch, S. M., Vuori, K., Ruoslahti, E. and Chan-Hui, P.-Y. (1996) Control of adhesion dependent cell survival by focal adhesion kinase. J. Biol. Chem. **134**, 793–799
33 Hungerford, J. E., Compton, M. T., Matter, M. L., Hoffstrom, B. G. and Otey, C. A. (1996) Inhibition of pp125FAK in cultured fibroblasts results in apoptosis. J. Biol. Chem. **135**, 1383–1390
34 Zhao, J.-H., Reiske, H. and Guan, J.-L. (1998) Regulation of the cell cycle by focal adhesion kinase. J. Cell Biol. **143**, 1997–2008

35 Hildebrand, J. D., Schaller, M. D. and Parsons, J. T. (1993) Identification of sequences required for the efficient localisation of the focal adhesion kinase, pp125$^{FAK}$, to cellular focal adhesions. J. Cell Biol. **123**, 993–1005
36 Tachibana, K., Sato, T., D'Avirro, N. and Morimoto, C. (1995) Direct association of pp125$^{FAK}$ with paxillin, the focal adhesion-targetting mechanism of pp125$^{FAK}$. J. Exp. Med. **182**, 1089–1099
37 Zachary, I., Sinnett-Smith, J., Turner, C. and Rozengurt, E. (1993) Bombesin, vasopressin and endothelin stimulate tyrosine phosphorylation of the focal adhesion-associated protein paxillin in Swiss 3T3 cells. J. Biol. Chem. **268**, 22060–22065
38 Calalb, M. B., Polte, T. R. and Hanks, S. K. (1995) Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for Src family kinases. Mol. Cell Biol. **15**, 954–963
39 Calalb, M. B., Zhang, X., Polte, T. R. and Hanks, S. K. (1996) Focal adhesion kinase tyrosine-861 is a major site of phosphorylation by Src. Biochem. Biophys. Res. Commun. **228**, 662–668
40 Rankin, S. and Rozengurt, E. (1994) Platelet-derived growth factor modulation of focal adhesion kinase (p125$^{FAK}$) and paxillin tyrosine phosphorylation in Swiss 3T3 cells. Bell shaped dose response and cross-talk with bombesin. J. Biol. Chem. **269**, 704–710
41 Abedi, H., Dawes, K. E. and Zachary, I. (1995) Differential effects of platelet-derived growth factor BB on p125 focal adhesion kinase and paxillin tyrosine phosphorylation and on cell migration in rabbit aortic vascular smooth muscle cells and Swiss 3T3 fibroblasts. J. Biol. Chem. **270**, 11367–11376
42 Schaller, M. D., Hildebrand, J. D., Shannon, J. D., Fox, J. W., Vines, R. R. and Parsons, J. T. (1994) Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60Src. Mol. Cel. Biol. **14**, 1680–1688
43 Schlaepfer, D. D., Hanks, S. K., Hunter, T. and van der Geer, P. (1994) Integrin-mediated signal transdcution linked to Ras pathway by GRB2 binding to focal adhesion kinase. Nature (London) **372**, 786–791
44 Ma, A., Richardson, A., Schaefer, E. M. and Parsons, J. T. (2001) Serine phosphorylation of focal adhesion kinase in interphase and mitosis: a possible role in modulating binding to p130$^{Cas}$. Mol. Biol. Cell **12**, 1–12
45 Polte, T. R. and Hanks, S. K. (1997) Complexes of focal adhesion kinase (FAK) and Crk-associated substrate (p130$^{Cas}$) are elevated in cytoskeleton-associated fractions following adhesion and Src transformation. Requirements for Src kinase activity and FAK proline-rich motifs. J. Biol. Chem. **272**, 5501–5509
46 Chen, H.-C. and Guan, J.-L. (1994) Association of focal adhesion kinase with its potential substrate phosphatidylinositol 3-kinase. Proc. Natl. Acad. Sci. U.S.A. **91**, 10148–10152
47 Chen, H.-C., Appendu, P. A., Parsons, J. T., Hildebrand, J. D., Schaller, M. D. and Guan, J.-L. (1995) Interaction of focal adhesion kinase with cytoskeletal protein talin. J. Biol. Chem. **270**, 16995–16999
48 Schaller, M. D., Otey, C. A., Hildebrand, J. D. and Parsons, J. T. (1995) Focal adhesion kinase and paxillin bind to peptides mimicking $\beta$ integrin cytoplasmic domains. J. Cell Biol. **130**, 1181–1187
49 Sieg, D. J., Hauck, C. R., Ilic, D., Klingbell, C. K., Schaefer, E., Damsky, C. H. and Schlaepfer, D. D. (2000) FAK integrates growth-factor and integrin signals to promote cell migration. Nat. Cell Biol. **2**, 249–256
50 Hauck, C. R., Hsia, D. A. and Schlaepfer, D. D. (2000) Focal adhesion kinase facilitates PDGF-BB-stimulated ERK2 activation required for chemotaxis migration of vascular smooth muscle cells. J. Biol. Chem. **275**, 41092–41099
51 Hanke, J. H., Gardner, J. P., Dow, R. L., Changelian, P. S., Brissette, W. H., Weringer, E. J., Pollok, B. A. and Connelly, P. A. (1996) Discovery of a novel, potent and Src-family-selective tyrosine kinase inhibitor. J. Biol. Chem. **271**, 695–701
52 Salazar, E. P. and Rozengurt, E. (1999) Bombesin and platelet-derived growth factor induce association of endogenous focal adhesion kinase with Src in intact Swiss 3T3 cells. J. Biol. Chem. **274**, 28371–28378
53 Lobo, M. and Zachary, I. (2000) Nuclear localization and apoptotic regulation of an amino-terminal domain focal adhesion kinase (FAK) fragment in endothelial cells. Biochem. Biophys. Res. Commun. **276**, 1068–1074
54 Levkau, B., Herren, B., Koyama, H. R., Ross, R. and Raines, E. W. (1998) Caspase-mediated cleavage of focal adhesion kinase pp125FAK and disassembly of focal adhesions in human endothelial cell apoptosis. J. Exp. Med. **187**, 579–586
55 van de Water, B., Nagelkerke, J. F. and Stevens, J. L. (1999) Dephosphorylation of focal adhesion kinase (FAK) and loss of focal contacts precede caspase-mediated cleavage of FAK during apoptosis in renal epithelial cells. J. Biol. Chem. **274**, 13328–13337
56 Ruoslahti, E. and Reed, J. (1994) Anchorage-dependence, integrins and apoptosis. Cell **77**, 477–478
57 Eliceiri, B. P., Paul, R., Schwartzberg, P. L., Hood, J. D., Leng, J. and Cheresh, D. A. (1999) Selective requirement for Src kinases during VEGF-induced angiogenesis and vascular permeability. Mol. Cell **4**, 915–924

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