NCK and PAK Participate in the Signaling Pathway by Which Vascular Endothelial Growth Factor Stimulates the Assembly of Focal Adhesions*

Received for publication, October 24, 2000, and in revised form, February 14, 2001
Published, JBC Papers in Press, March 8, 2001, DOI 10.1074/jbc.M009720200

Konstantin V. Stoletov, Kirsty E. Ratcliffe, Simone C. Spring, and Bruce I. Terman‡
From the Departments of Medicine and Pathology, Albert Einstein College of Medicine, Bronx, New York 10461

Vascular endothelial growth factor (VEGF)-induced endothelial cell migration is a key step in the angiogenic response and is mediated, in part, by an accelerated rate of focal adhesion complex assembly and disassembly. We investigated the signaling pathway by which VEGF regulates focal adhesion complex assembly by examining the signaling proteins involved. VEGF stimulated the tyrosine phosphorylation of the SH2 domain-containing signaling proteins NCK and CRK in human umbilical vein endothelial cells. The signaling pathways that couple the kinase insert domain-containing receptor to NCK and CRK is most likely mediated by another cellular protein, as NCK and CRK were tyrosine-phosphorylated in response to VEGF in cells expressing receptors mutated at each of several candidate SH2 domain-interacting cytosolic tyrosines. In the absence of VEGF treatment, NCK (but not CRK) associated with the p21 GTPase-activated kinase PAK. PAK catalytic activity was augmented after VEGF treatment; an association of PAK with 60- and 90-kDa tyrosine-phosphorylated proteins accompanied this. VEGF stimulated the recruitment of PAK to focal adhesions, and PAK immunoprecipitated with both NCK and PAK in VEGF-treated (but not untreated) human umbilical vein endothelial cells. Inhibition of NCK protein expression using antisense oligonucleotides led to the inhibition of both VEGF-induced focal adhesion assembly and VEGF-induced cell migration, demonstrating a necessary role of NCK in these cellular responses.

Angiogenesis, the formation of new blood capillaries, is an important component of normal physiological processes such as wound healing and development (reviewed in Ref. 1). Angiogenesis also contributes to several pathological situations, including tumor growth (2, 3), rheumatoid arthritis (4), and degenerative eye disorders (5, 6). Angiogenesis is a complex process involving endothelial cell movement and proliferation and endothelial cell-mediated degradation of the extracellular matrix. Multiple angiogenic stimulators and inhibitors regulate these processes (7–9).

Vascular endothelial growth factor (VEGF)1 has received attention as a key regulator of endothelial cell functions (10–12). VEGF expression correlates both temporally and spatially with the onset of angiogenesis in several physiological situations (13–15), and VEGF elicits a strong angiogenic response in a variety of in vivo models (16, 17). An essential role of VEGF in tumor angiogenesis and ischemia-related retinal disorders has been demonstrated by the findings that neutralizing anti-VEGF antibodies or dominant-negative VEGF receptors inhibit both angiogenesis and the progression of these pathological disorders (18–21).

A major component of the angiogenic response are the changes that occur in endothelial cell interactions with the extracellular matrix as well as changes in cell-cell interactions. Endothelial cells are linked to each other by tight- and adherens-type junctions and are linked to the extracellular matrix by a variety of integrin and other adhesion molecules (22–25). VEGF activates endothelial cells, in part, through stimulating signal transduction pathways that regulate the enzymatic components of adhesion complexes. VEGF-induced tyrosine phosphorylation of VE-cadherins (26), a component of adherens-type cell-cell junctions, has been implicated as a key step in endothelial cell migration. VEGF-induced phosphorylation of the tight junction proteins occludin and zonula occluden-1 (27, 28) is a potential mechanism by which the growth factor enhances vascular permeability. Experimental evidence indicating a role for VEGF in regulating cell-matrix interactions includes the findings that VEGF enhances the expression of α1β1 and α2β1 integrins (29) and that neutralizing antibodies to α, β integrins block growth factor-induced neovascularization (30, 31).

VEGF exhibits high affinity binding to two distinct receptor tyrosine kinases, the fms-like tyrosine kinase Flt-1 (32, 33) and the kinase insert domain-containing receptor (KDR) (34, 35). Both receptors possess insert sequences within their catalytic domains and seven immunoglobulin-like domains in the extracellular regions and are related to the platelet-derived growth factor family of receptor tyrosine kinases. Although expression of both VEGF receptor types occurs in adult endothelial cells, including human umbilical vein endothelial (HUVE) cells, recent findings suggest that KDR, and not Flt-1, is able to mediate the mitogenic and chemotactic effects of VEGF in endothelial cells (36). The key targets that mediate the diverse biological functions of VEGF in endothelial cells remain incompletely understood for either VEGF receptor. Somewhat varying results as to the downstream effects for the VEGF receptors...
have been obtained. VEGF stimulates the tyrosine phosphorylation of phospholipase Cγ, MAPK, phosphatidylinositol 3-kinase, FAK, and paxillin in HUVE cells and of phospholipase Cγ, p120GAP, and NCK in bovine aortic endothelial cells (37, 38). In porcine aortic endothelial cells transfected with KDR and Flt-1, VEGF has no effect on phosphatidylinositol 3-kinase activity and only a weak effect on p120GAP tyrosine phosphorylation (39).

The goal of this study was to clarify the signaling mechanism by which VEGF stimulates endothelial cell migration. As there is compelling evidence that the assembly and disassembly of focal adhesions play a key role in the mechanism by which several extracellular stimuli regulate both cell morphology and movement (40–42), and VEGF stimulates the tyrosine phosphorylation of FAK (37), we took the strategy of exploring the cell signaling proteins that couple VEGF binding to its receptors with focal adhesion assembly. Our results demonstrate that the SH2 domain-containing signaling protein NCK and the p21 GTPase-activated serine/threonine kinase PAK play necessary roles in this signaling pathway.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HUVE cells were isolated as previously reported (43).

Passage 2 cells were cultured on 0.2% gelatin-coated tissue culture plates in medium 199 containing 20% newborn calf serum, 5% human serum, and 7.5 μg/ml endothelial growth factor (Sigma).

**Antibodies**—The rabbit anti-KDR antibody was isolated in our laboratory and targets a polypeptide domain within the KDR cytosolic domain (44). Anti-NCK, anti-CRK, anti-FAK, and anti-phosphotyrosine (PY20) monoclonal antibodies were from Transduction Laboratories. Anti-FAK and anti-PAK polyclonal antibodies were from Santa Cruz Biotechnology, Inc. Peroxidase-conjugated donkey anti-rabbit and sheep anti-mouse immunoglobulins were from Amersham Pharmacia Biotech.

**Western Blotting**—HUVE cells were grown on 10-cm dishes until subconfluent. The cells were incubated in serum-free DMEM with 1 mM Na$_2$VO$_4$, 0.1 mM NaCl, 50 mM Na$_3$VO$_4$, 30 mM Na$_4$P$_2$O$_7$, 30 mM NaF, 1 mM EDTA, 1 mg/ml leupeptin, 0.7 mg/ml pepstatin A, and 1% Triton X-100, and primary antibodies were added to the lysates. After a 1-h incubation at 4 °C, the antibodies were immobilized on protein A-Sepharose beads (Sigma), and the beads were washed three times with lysis buffer and boiled in 40 μl of SDS-polyacrylamide gel electrophoresis sample loading buffer. Proteins were separated on SDS-polyacrylamide gels, transferred to nitrocellulose and incubated for 1 h at room temperature. The filters were then blocked in Tris-buffered saline containing 0.2% Tween plus 5% dry milk at 4 °C overnight and probed with appropriate antibodies. The filters were visualized through enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

**Site-directed Mutagenesis of the KDR cDNA**—For substitution of tyrosine residues with phenylalanine, site-directed mutagenesis was carried out on KDR cDNA using the U.S.E. mutagenesis kit (Amersham Pharmacia Biotech.). The following oligonucleotides were used for mutagenesis: 5′-TTGCTTCAAAGACTTCTTCTCC-3′ (Y951F), 5′-GGAGAGTCTCTAACAAGACTTCTA-3′ (Y969F), 5′-TTACCCCGAGGAATGG-3′ (Y1106F), 5′-CTGGTGTTTATCATCAGGGCC-3′ (Y1130F), and 5′-GGAGAACGAACTTCTTCTCC-3′ (Y1175F). All mutations were confirmed by DNA sequencing. The resulting vectors were transfected into HEK293 cells, and stable transfectants were selected and expanded as previously described (44).

**In Vitro Kinase Assay**—HUVE cells were grown on 10-cm dishes until subconfluent. Cells were treated with VEGF (50 ng/ml) for 15 min at 37 °C. Cells were harvested with ice-cold serum-free medium, and primary antibodies were added to the lysates. After a 1-h incubation at 4 °C, the antibodies were immobilized on protein A-Sepharose beads (Sigma), and the beads were washed three times with lysis buffer and boiled in 40 μl of SBS-polyacrylamide gel electrophoresis sample loading buffer. Proteins were separated on SDS-polyacrylamide gels, transferred to nitrocellulose and incubated for 1 h at room temperature. The filters were then blocked in Tris-buffered saline containing 0.2% Tween plus 5% dry milk at 4 °C overnight and probed with specific antibodies for 2 h at room temperature. After washing, the filter was incubated with horseradish peroxidase-linked anti-rabbit or anti-mouse IgG, and reactions were visualized through enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

**RESULTS**

**VEGF Stimulates the Recruitment of NCK and CRK to KDR**—It was previously shown that NCK tyrosine phosphorylation and the amount of NCK present in anti-KDR immunoprecipitates are increased in VEGF-treated bovine aortic endothelial cells and KDR-transfected porcine aortic endothelial cells (38, 39). The experimental data shown in Fig. 1 (A and B) demonstrate that similar results were obtained using HUVE cells.

NCK is a member of a family of cell proteins that lack enzymatic activity and that participate in cell signaling by acting as adaptor proteins (45). Fig. 1C demonstrates that VEGF treatment of HUVE cells enhanced the tyrosine phosphorylation of another adaptor protein, CRK. The 42-kDa isoform (CRKII), but not 28-kDa CRKI, was detected in the anti-KDR immunoprecipitates after VEGF treatment, even though both isoforms are expressed in these cells.

Experiments were performed to determine if the interaction of NCK and CRK with VEGF-activated KDR is direct or mediated through another protein. The strategy was to express receptor protein in which specific tyrosines had been mutated to phenylalanine in HEK293 cells (which do not express endog-
Subconfluent HUVE cells were incubated for 1 h in serum-free DMEM containing 1 mM Na3VO₄ and then with or without 50 ng/ml VEGF for the times indicated (A) or for 20 min (B and C) at 4 °C. The cells were lysed, and anti-Tyr(P) (pTyr) (A and C) or anti-KDR (B) immunoprecipitates (IP) were prepared and immunoblotted (IB) with anti-NCK (A and B) or anti-CRK (C) antibodies. Arrows in A and B indicate the expected positions of NCK. The molecular masses given in C were derived from standards run on the gel shown. The numbers indicate the fold stimulation caused by VEGF of the intensity of the NCK and CRK proteins as determined using a PhosphorImager. The LYSATE lane in C is derived from HUVE cells boiled in gel sample buffer and loaded directly on the gel.

To validate the experimental strategy, we also examined VEGF-induced NCK and CRK tyrosine phosphorylation in HEK293 cells expressing native and mutant KDRs. A, cells expressing native or mutant KDR were grown to confluence on 24-well dishes. The cell medium was aspirated, and 200 μl of gel sample buffer was added to the wells. The samples were transferred to Eppendorf tubes and boiled for 5 min, and 25 μl was subjected to 7% SDS-polyacrylamide gel electrophoresis. Immunoblotting was done using anti-KDR antibodies. B–C, cells expressing native or mutant KDR were grown to confluence on 10-cm dishes and then incubated overnight in DMEM containing 1% fetal calf serum. After a 1-h incubation in serum-free DMEM containing 1 mM Na3VO₄, the cells were incubated at 4 °C for 4 h with or without 50 ng/ml VEGF. The cells were lysed, and anti-Tyr(P) (pTyr) immunoprecipitates (IP) were prepared and immunoblotted (IB) with anti-KDR (B), anti-NCK (C), or anti-CRK (D) antibodies. Aliquots of cells not immunoprecipitated with antibody were loaded on the SDS-polyacrylamide gels and are indicated as LYSATE. The experiment done for A was different that those done for B–D. Results identical to those shown in B–D were observed in three separate experiments.

Vegetative NCK and CRK phosphorylation was induced by VEGF in HUVE cells. B, an increased amount of NCK and CRK was immunoprecipitated with anti-KDR antibodies. C, results identical to those shown in B–D were observed in three separate experiments.

![Image](https://example.com/image1.png)

**Fig. 1.** VEGF stimulates NCK and CRK tyrosine phosphorylation and association of NCK and CRK with KDR in HUVE cells. Subconfluent HUVE cells were incubated for 1 h in serum-free DMEM containing 1 mM Na3VO₄ and then with or without 50 ng/ml VEGF for the times indicated (A) or for 20 min (B and C) at 4 °C. The cells were lysed, and anti-Tyr(P) (pTyr) (A and C) or anti-KDR (B) immunoprecipitates (IP) were prepared and immunoblotted (IB) with anti-NCK (A and B) or anti-CRK (C) antibodies. Arrows in A and B indicate the expected positions of NCK. The molecular masses given in C were derived from standards run on the gel shown. The numbers indicate the fold stimulation caused by VEGF of the intensity of the NCK and CRK proteins as determined using a PhosphorImager. The LYSATE lane in C is derived from HUVE cells boiled in gel sample buffer and loaded directly on the gel.

![Image](https://example.com/image2.png)

**Fig. 2.** NCK and CRK tyrosine phosphorylation in HEK293 cells expressing native and mutant KDRs. A, cells expressing native or mutant KDR were grown to confluence on 24-well dishes. The cell medium was aspirated, and 200 μl of gel sample buffer was added to the wells. The samples were transferred to Eppendorf tubes and boiled for 5 min, and 25 μl was subjected to 7% SDS-polyacrylamide gel electrophoresis. Immunoblotting was done using anti-KDR antibodies. B–C, cells expressing native or mutant KDR were grown to confluence on 10-cm dishes and then incubated overnight in DMEM containing 1% fetal calf serum. After a 1-h incubation in serum-free DMEM containing 1 mM Na3VO₄, the cells were incubated at 4 °C for 4 h with or without 50 ng/ml VEGF. The cells were lysed, and anti-Tyr(P) (pTyr) immunoprecipitates (IP) were prepared and immunoblotted (IB) with anti-KDR (B), anti-NCK (C), or anti-CRK (D) antibodies. Aliquots of cells not immunoprecipitated with antibody were loaded on the SDS-polyacrylamide gels and are indicated as LYSATE. The experiment done for A was different that those done for B–D. Results identical to those shown in B–D were observed in three separate experiments.

Vegetative NCK and CRK phosphorylation was induced by VEGF in HUVE cells. B, an increased amount of NCK and CRK was immunoprecipitated with anti-KDR antibodies. C, results identical to those shown in B–D were observed in three separate experiments.

![Image](https://example.com/image3.png)

**Fig. 3.** VEGF-induced NCK and SHC activation in HEK293 cells expressing native and mutant KDRs. The experiments were done exactly as described for Fig. 2 (B–D), except that immunoprecipitation (IP) was with anti-KDR (A and B) or anti-Tyr(P) (pTyr) (C) antibodies, and immunoblotting (IB) was done with anti-NCK (A) or anti-SHC (C) antibodies. The blot shown in A was reprobed with the anti-KDR antibody (B) to ensure that equal amounts of the immunoprecipitates were loaded for the control and VEGF-treated samples. Results identical to those shown in each panel were observed in three separate experiments.

Vegetative NCK and CRK phosphorylation was induced by VEGF in HUVE cells. B, an increased amount of NCK and CRK was immunoprecipitated with anti-KDR antibodies. C, results identical to those shown in B–D were observed in three separate experiments.

![Image](https://example.com/image4.png)

**Fig. 4.** VEGF-induced NCK and SHC activation in HEK293 cells expressing native and mutant KDRs. The experiments were done exactly as described for Fig. 2 (B–D), except that immunoprecipitation (IP) was with anti-KDR (A and B) or anti-Tyr(P) (pTyr) (C) antibodies, and immunoblotting (IB) was done with anti-NCK (A) or anti-SHC (C) antibodies. The blot shown in A was reprobed with the anti-KDR antibody (B) to ensure that equal amounts of the immunoprecipitates were loaded for the control and VEGF-treated samples. Results identical to those shown in each panel were observed in three separate experiments.

Vegetative NCK and CRK phosphorylation was induced by VEGF in HUVE cells. B, an increased amount of NCK and CRK was immunoprecipitated with anti-KDR antibodies. C, results identical to those shown in B–D were observed in three separate experiments.
phosphorylation of myelin basic protein using cell lysates. PAK activity was measured by monitoring the activity of PAK. HUVE cells were treated with or without VEGF, and anti-PAK immunoprecipitates were prepared from cell extracts. Experiments were then done to determine whether PAK participates in VEGF-induced signal transduction by acting downstream from NCK. Western blot analysis demonstrated the presence of PAK in anti-NCK immunoprecipitates (Fig. 4A), but not anti-CRK immunoprecipitates (data not shown), isolated from HUVE cell extracts. The anti-PAK antibody recognizes at least three PAK isoforms (α-PAK (PAK1), β-PAK (PAK3), and γ-PAK (PAK2)), and multiple isoforms were detected after immunoprecipitation with NCK antisera. VEGF had no effect on the amount of PAK associated with NCK (Fig. 4A); this conclusion was confirmed by a complementary experiment in which cell extracts were immunoprecipitated with the anti-PAK antibody, and Western blotting was performed with the anti-NCK antibody (Fig. 4B).

These results indicate that PAK is bound to NCK, even in the absence of growth factor. Experiments were then done to determine the consequence of VEGF treatment on the enzymatic activity of PAK. HUVE cells were treated with or without VEGF, and anti-PAK immunoprecipitates were prepared from cell lysates. PAK activity was measured by monitoring the phosphorylation of myelin basic protein using $\gamma^{-32}$P]ATP, SDS-polyacrylamide gel electrophoresis, and autoradiography. As shown in Fig. 5A, the phosphorylation of myelin basic protein was significantly increased in samples from VEGF-treated compared with untreated cells.

$32$P labeling of one other protein (60 kDa) was enhanced in anti-PAK immunoprecipitates prepared from the VEGF-treated samples (Fig. 5A). Proteins of 60 and 90 kDa were observed upon blotting anti-PAK immunoprecipitates with the anti-Tyr(P) antibody (Fig. 5B). The identities of the 90- and 60-kDa proteins are not known, and neither is the tyrosine kinase responsible for their phosphorylation.

It was previously shown that the expression of a constitutively active PAK leads to recruitment of the recombinant protein to focal adhesions (51, 52). We therefore tested whether the VEGF-activated PAK. In the absence of VEGF, immunofluorescent staining with the anti-PAK antibody (Fig. 6C) revealed no focal adhesion-like structures. Focal adhesions, as observed using anti-FAK (Fig. 6A) or anti-Tyr(P) (data not shown) antibodies, were present in these cells, but they were $25\%$ the size of those seen in the presence of VEGF (Fig. 6B). Within 15 min of VEGF treatment, PAK staining (Fig. 6D) was identical to the staining with the anti-FAK or anti-Tyr(P) antibody, demonstrating the recruitment of PAK to focal adhesions.

Both the KDR and Flt-1 VEGF receptor subtypes are expressed by HUVE cells. Although it was previously demonstrated that VEGF-induced endothelial cell migration is mediated by KDR and not Flt-1 (36), it cannot be ruled out that Flt-1 does not participate in the effects of VEGF on PAK activation and focal adhesion assembly. To address this issue, we asked whether PIGF treatment of HUVE cells leads to focal adhesion assembly. The rationale for this experiment was that PIGF, which shares $50\%$ amino acid homology with VEGF, binds to Flt-1, but not KDR. The results shown in Fig. 7 demonstrate that immunofluorescent staining of PIGF-treated cells (Fig. 7B) using the anti-PAK antibody was identical to that observed for control cells not treated with growth factor (Fig. 7A).

Further evidence indicating that VEGF stimulates the recruitment of PAK to focal adhesions is shown in Fig. 8. The results demonstrate that PAK was detected in both anti-PAK (Fig. 8A) and anti-NCK (Fig. 8D) immunoprecipitates obtained from VEGF-treated (but not untreated) HUVE cell extracts.

**NCK Antisense Oligonucleotides Block VEGF-induced Focal Adhesion Formation and Cell Migration**—The experimental results shown in Figs. 6 and 7 demonstrate that VEGF treatment leads to NCK and PAK localization to focal adhesions, but...
do not clarify the functional requirement of these signaling proteins in either the formation of focal adhesions or biological activities associated with these cellular complexes. To address these issues, we took the strategy of inhibiting NCK expression using antisense oligonucleotides and testing the consequence on the formation of focal adhesions. A 19-mer 2′-O-methyl oligoribonucleoside phosphorothioate targeting the NCK translation initiation site (codons 2–7) was used. The corresponding sense oligonucleotide was used as a control. The transfection protocol utilized LipofectAMINE and resulted in a fluorescein-labeled oligonucleotide (data not shown). The transfection protocol using either sense or antisense oligonucleotide (0.4 μmol/ml) had only a small effect on the viability of the cells compared with cells subjected to transfection with no oligonucleotide, with a <10% loss in cell number after 3 days. There was a significant (25%) increase in the number of rounded cells 3 days post-transfection with either antisense or sense oligonucleotide compared with mock-transfected cells, indicating that the oligonucleotides do have some nonspecific effects.

The amount of cellular NCK protein was determined at several time points following transfection. From analyzing several experiments (n = 6; 3 days post-transfection), we found that the level of NCK protein was reduced between 60 and 80% by antisense oligonucleotides compared with cells subjected to the protocol without oligonucleotides (Fig. 9A). There was also a 20–25% decrease in the level of NCK protein after transfection with sense oligonucleotides (Fig. 9A). As a control, we compared the level of CRK protein in NCK antisense versus NCK sense oligonucleotide-transfected cells (Fig. 9B) and found no differences in any of the experiments.

Prior transfection of HUVE cells with sense oligonucleotide had no effect on VEGF activation of focal adhesion formation as determined by immunofluorescent staining with anti-FAK antibodies (Fig. 10, A and B). As is the case with cells not subjected to the transfection protocols, cells transfected with sense oligonucleotides and not treated with VEGF contained small focal adhesions, and VEGF caused an increase in both their number and size. VEGF-induced redistribution of PAK to focal adhesions was also not affected in the sense oligonucleotide-transfected cells (Fig. 10, C and D). In the absence of VEGF treatment, cells transfected with the antisense oligonucleotides contained a similar number of the small focal adhesions seen in cells not transfected or transfected with sense oligonucleotide. However, in the presence of VEGF, transfection with the NCK antisense oligonucleotides significantly reduced the number of large focal adhesions seen in control cells (Fig. 10, E and F).

VEGF stimulates the directed migration of HUVE cells, and...
FAK has been implicated as playing a necessary role in this response (40). In view of our data (Fig. 10) demonstrating a necessary role of NCK in focal adhesion formation, we examined whether inhibition of NCK using antisense oligonucleotides would block VEGF-induced migration. The results shown in Fig. 11 confirm that this is in fact the case. HUVE cells transfected with NCK antisense oligonucleotides were significantly less responsive to VEGF than either mock-transfected or NCK sense oligonucleotide-transfected cells. These results demonstrate that VEGF signaling through NCK is required for endothelial cell migration.

**DISCUSSION**

In this study, we describe a signal transduction pathway that couples VEGF binding to its receptor with focal adhesion formation and cell migration. The model predicts that VEGF binding to KDR leads to the recruitment of an NCK-PAK complex to the receptor, with subsequent tyrosine phosphorylation of NCK. PAK catalytic activity is then augmented, accompanied by increased phosphorylation of 60- and 90-kDa proteins. The NCK-PAK complex is recruited to focal adhesions, which is followed by enhancement of cell migration. Each of these steps is now discussed in greater detail.

HUVE cells express both the KDR and Flt-1 VEGF receptor subtypes, and NCK associates with both receptors after VEGF treatment (55). Although Fig. 7 shows that PlGF treatment of these cells does not stimulate focal adhesion assembly, it is not clear whether PlGF-induced Flt-1 signaling is identical to that for VEGF. Further evidence consistent with the conclusion that VEGF binding to KDR mediates the effects described in this study is the fact that Flt-1 does not participate in VEGF-induced cell migration (36) and results demonstrating that VEGF treatment of KDR-transfected porcine aortic endothelial cells (which do not express Flt-1) leads to both NCK tyrosine phosphorylation and an increase in focal adhesion assembly (53).
It was concluded from the data in Figs. 3 and 4 that NCK and CRK do not interact directly with KDR after VEGF treatment or interact with the receptor at a previously not recognized peptide sequence. The tyrosines that were targeted for mutagenesis in these experiments were chosen based upon whether their adjacent amino acid sequences conformed to a consensus-binding site (pYDE(P/D/V)) for the NCK SH2 domain (49, 50). Neither of the known NCK-binding sites on the platelet-derived growth factor receptor tyrosine kinase kinase (SVDY-pYVPMLD) or Flt-1 (SVVLpYSTPPI) contains this consensus recognition sequence, which indicates that there is binding promiscuity. None of the 18 tyrosines within the cytosolic domain of KDR are contained within peptides conforming to this sequence. The KDR peptide that most closely matches the consensus amino acid sequence is RAPDY1130TTPE, and other peptides sequence. The KDR peptide that most closely matches the consensus-binding site (pYDE(P/D/V)) for the NCK SH2 domain. None of the 18 tyrosines within the cytosolic domain of KDR are contained within peptides conforming to this sequence.

The hypothesis that the binding of NCK with KDR is indirect is consistent with evidence that has been reported for the epidermal growth factor receptor (54, 55), in which the binding of epidermal growth factor leads to the recruitment of the Dok-related (DokR) protein to the epidermal growth factor receptor, with subsequent binding of NCK to DokR. We did not detect DokR in anti-NCK immunoprecipitates prepared from cell lysates after VEGF treatment (data not shown). Other receptor tyrosine kinases bind NCK directly after growth factor activation. For example, VEGF binding to the Flt-1 receptor subtype leads to a direct interaction of both NCK and CRK with Flt-1 Tyr(P)1333 (56). The amino acid sequence containing Flt-1 Tyr1333 (SVVLpYSTPPI) is quite similar to the sequence containing KDR Tyr1130 (RAPDY1130TTPE), but we conclude that there are sufficient structural differences so that NCK does not bind to the KDR peptide.

An interaction of NCK with PAK in quiescent cells and the ability of activated growth factor (e.g. platelet-derived growth factor and epidermal growth factor) receptors to recruit the complex to the cells’ surface have been noted previously (57, 58). The association between NCK and PAK is mediated by the second SH3 domain of NCK and a proline-rich sequence in the amino terminus of PAK (58, 59). Our finding that PAK activity is enhanced by VEGF is consistent with previous studies documenting that localization of the second NCK SH3 domain, or of PAK itself, to the cell-surface membrane results in PAK activation; this is independent of other signals. These previous studies showed that PAK activation by a membrane-targeted NCK SH3 domain is blocked by negative regulators of the Cdc42 or Rac GTP-binding proteins (60, 61). The activation of PAK by VEGF treatment is therefore likely to involve Cdc42 or Rac, although G-protein-independent mechanisms of PAK activation have also been reported (62).

The VEGF-stimulated increase in PAK activity is accompanied by the phosphorylation of 60- and 90-kDa proteins. Both of these proteins associate with PAK as demonstrated by their immunoprecipitation using anti-PAK antibodies. The PAK family of serine/threonine kinases ranges in molecular mass from 62 to 68 kDa (63, 64), and PAK autoprophosphorylation is an important mechanism by which PAK cellular function is regulated (65). The 60-kDa protein seen in Fig. 5A is most likely not a PAK family member because it is tyrosine-phosphorylated in response to VEGF. We cannot rule out the possibility that the [32P]ATP phosphorylation of the 60-kDa protein is due to a tyrosine kinase that immunoprecipitates with the anti-PAK antibody, and not PAK itself. The identity of the tyrosine-phosphorylated 90-kDa PAK substrate is also not known, but most likely it is the 90-kDa protein identified in anti-PAK immunoprecipitates by other investigators (57, 66).

It is not known whether the catalytic activity of PAK participates in either focal adhesion assembly/disassembly or if it has some other role in VEGF-induced signaling. Studies from the literature have yielded seemingly conflicting results on the precise roles that the PAKs play in regulating reorganization of the actin cytoskeleton. For example, Cdc42 and Rac mutants defective for PAK binding can still form filopodia and lamellipodia (67, 68), suggesting that PAK activity is not required for these effects. On the other hand, an activated PAK1 mutant that is defective in Cdc42 or Rac binding promotes formation of polarized membrane ruffles and vinculin-containing focal adhesions in Swiss 3T3 cells (51, 52). This effect of PAK is independent of its kinase activity. The confusion regarding the precise role of PAK in Cdc42- and Rac-dependent morphological changes has been partially resolved by the finding that a family of PAK-binding proteins (PIX and COOL) exists that acts to enhance the coupling between Cdc42 and PAK and is required for PAK recruitment to focal adhesions (66).

Our finding that NCK participates in the signal transduction pathway by which VEGF stimulates endothelial cells to migrate is consistent with the results obtained by Kiosses et al. (69). These authors studied the role of PAK in endothelial cell migration after activating endogenous PAK either by replating serum-starved cells on fibronectin or by microinjecting cells with recombinant catalytically active or dominant-negative protein. The conclusion of the study was that PAK plays a role in coordinating leading edge adhesion formation and trailing edge detachment to produce polarized cell movement. Significantly, the role of PAK in cell migration was found to be dependent upon a short proline-rich sequence that is known to bind the SH2 domain of NCK, as a recombinant PAK lacking this domain acted as a dominant-negative inhibitor of cell migration after microinjection into endothelial cells.

Acknowledgments—We thank Dr. Anthony Ashton for valuable assistance with the cell migration assays and Dona Wu, Harry Ma, Tina Calderon, and Lillie Lopez for assistance with culturing HUVE cells.

REFERENCES

1. Folkman, J., and Shing, Y. (1992) J. Biol. Chem. 267, 10931–10934
2. Folkman, J. (1991) J. Natl. Cancer Inst. 82, 4–6
3. Yoshiji, H., Gomez, D. E., Shibuya, M., and Thorgeirsson, U. P. (1996) Cancer Res. 56, 2013–2015
4. FBI, J. T., Brown, N. J., Spencer-Green, G., Yeo, T.-K., Yeo, K.-T., Berse, B., Jackman, R. W., Senger, D. R., Dvorak, H. F., and Brown, L. F. (1994) J. Exp. Med. 180, 340–346
5. Peré, J., Shweiki, D., Itin, A., Hemo, I., Gnessin, H., and Keshet, E. (1995) Lab. Invest. 72, 638–645
6. Pierce, F. A., Avery, R. L., Foley, E. D., Aiello, L. P., and Smith, L. E. H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 905–909
7. O'Reilly, M. S., Boehm, T., Shing, Y., Kukai, N., Vasios, G., Lane, W. S., Flynn, W., and Folkman, J. (1997) Cell 88, 277–285
8. Brewer, T., Folkman, J., and Pirie-Shipard, S. (2000) J. Biol. Chem. 275, 1521–1524
9. Davis, S., Aldrich, T. H., Jones, P. F., Acheson, A., Compton, D. L., Jain, V., Ryan, T. E., Bruno, J., Radziejewski, C., Maisonneuve, P. C., and Younghoe, G. D. (1994) Cell 77, 1161–1169
10. Keck, P. M., Hauser, S. D., Krivi, G., Sanzo, K., Warren, T., Feder, J., and Connolly, D. T. (1989) Science 246, 1309–1312
11. Leung, D. W., Cachianes, G., Kuang, W. J., Goeddel, D. V., and Ferrara, N. (1989) Science 246, 1309–1312
12. Ferrara, N., and Davis-Smith, T. (1997) Endocr. Rev. 18, 4–25
13. Li, J., Brown, L. F., Hibberd, M. G., Grossman, J. D., Morgan, J. P., and Simons, M. (1996) Am. J. Physiol. 270, H1803–H1811
14. Shweiki, D., Itin, A., Soffer, D., and Keshet, E. (1992) Nature 359, 843–845
15. Brown, L. F., Berse, B., Jackman, R. W., Tomazycz, K., Maneau, E. J., Senger, D. R., and Dvorak, H. F. (1993) Am. J. Pathol. 143, 1255–1262
16. Takeshita, S., Zheng, L., Brugi, E., Kearney, M., Pu, L.-Q., Bunting, S., Ferrara, N., Simmes, J. F., and Isner, J. M. (1994) J. Clin. Invest. 93, 662–670
17. Majesky, M. W. (1996) Circulation 94, 3061–3064
18. Kim, K. F., Li, B., Winer, J., Armanini, M., Gillett, N., Philips, H. S., and Ferrara, N. (1993) Nature 366, 841–844
19. Milauza, B., Shawer, L. K., Plate, K. H., Risau, W., and Ullrich, A. (1994)
Nature 367, 576–579
20. Aiello, L. P., Pierce, E. A., Foley, E. D., Takagi, H., Chen, H., Ruddle, L., Ferrara, N., King, G. L., and Smith, L. E. H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10457–10461
21. Honda, M., Sakamoto, T., Ishibashi, T., Inomata, H., and Ueno, H. (2000) Gene Ther. 7, 978–985
22. Mitic, L. L., and Anderson, J. M. (1998) Annu. Rev. Physiol. 60, 121–142
23. Gumbiner, B. M. (1996) Cell 84, 345–357
24. Carmeliet, P., Lampugnani, M.-G., Moons, L., Breviario, F., Compernolle, V., Bone, P., Balconi, G., Spagnuolo, R., Oestuyse, G., Dewerchin, M., Zanetti, A., Angelilli, A., Mattot, V., Nuyens, D., Lutgens, E., Clotman, F., de Ruiter, M. C., Gitterberger-de Groot, A., Poelmann, R., Lupu, F., Herbert, J. M., Collen, D., and Dejana, E. (1999) Cell 96, 147–157
25. Brooks, P. C., Clark, R. A. F., and Cheresh, D. A. (1994) Science 264, 569–572
26. Esser, S., Lampugnani, M.-G., Corada, M., Dejana, E., and Risau, W. (1998) J. Cell Sci. 111, 1853–1865
27. Kevil, C. G., Payne, D. K., Mire, E., and Alexander, J. S. (1998) J. Biol. Chem. 273, 15099–15103
28. Antonetti, D. A., Barber, A. J., Hällinger, L. A., Wolpert, E. B., and Gardner, T. W. (1999) J. Biol. Chem. 274, 23462–23467
29. Senger, D. R., Clafley, K. P., Benes, J. E., Perruzzi, C. A., Sergiou, A. P., and Furcht, L. T. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7186–7190
30. Friedlander, M., Brooks, P. C., Shaffer, R. W., Kincaid, C. M., Varner, J. A., and Cheresh, D. A. (1995) Science 270, 1500–1502
31. Soldi, R., Mitola, S., Stralsy, M., Defilippi, P., Tarone, G., and Bussolino, F. (1999) EMBO J. 18, 882–892
32. Shihuya, M., Yamaguchi, S., Yamane, A., Ikeda, T., Tojo, A., Hitoshi, M., and Sato, M. (1990) Oncogene 4, 2079–2089
33. De Vries, C., Escobedo, J. A., Lechleider, R. J., Chant, J., and Hall, A. (1996) J. Cell Sci. 107, 1579–1586
34. Keyt, B., Nguyen, H., Berleau, L., Buarte, C., Park, J., Chen, H., and Ferrara, N. (1996) J. Biol. Chem. 271, 5638–5644
35. Abedi, H., and Zachary, I. (1997) J. Biol. Chem. 272, 15442–15451
36. Guo, D., Jia, Q., Song, H.-Y., Warren, R. S., and Donner, D. B. (1995) J. Biol. Chem. 270, 6729–6733
37. Waltenberger, J., Claesson-Welsh, L., Sieghahn, A., Shibuya, M., and Heldin, C. H. (1994) J. Biol. Chem. 269, 26988–26995
38. Gilmore, A. P., and Romer, L. H. (1996) Mol. Biol. Cell 7, 1209–1224
39. Bokoch, G. M., Wang, Y., Bohl, B. P., Sells, M. A., Quilliam, L. A., and Knaus, U. G. (1996) J. Biol. Chem. 271, 25746–25749
40. Kiosses, W. B., Daniels, R. H., Otey, C., Bokoch, G. M., and Schwartz, M. A. (1999) Oncogene 18, 797–806
41. Manser, E., Leung, T., Salhuddin, H., Zhao, Z.-S., and Lim, L. (1994) Nature 367, 40–46
42. Bagrodia, S., and Cerione, R. A. (1999) Trends Cell Biol. 9, 350–355
43. Zhao, Z.-S., Manser, E., and Lim, L. (2006) Mol. Cell. Biol. 26, 3906–3917
44. Manfra, M., Soehn, H. C., Zhao, Z.-S., Chen, X.-Q., Tan, L., Tan, L., Leung, T., and Lim, L. (1998) Mol. Cell 1, 183–192
45. Lamarche, N., Tapon, N., Stowers, L., Burke, P. D., Aspenstrom, P., Bridges, T., Chant, J., and Hall, A. (1998) Cell 87, 519–529
46. Yang, Z., Zhao, Y., Yang, Z., Wang, Y., and Bokoch, G. M. (1998) J. Biol. Chem. 273, 15125–15131
47. Legrand, J., Kiosses, W. B., Daniels, R. H., Otey, C., Bokoch, G. M., and Schwartz, M. A. (1999) J. Biol. Chem. 274, 8137–8144