Phosphorylation of Tyr$^{1214}$ within VEGFR-2 Triggers the Recruitment of Nck and Activation of Fyn Leading to SAPK2/p38 Activation and Endothelial Cell Migration in Response to VEGF*

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VEGFR-2 is the major receptor that regulates the different functions of VEGF in adults. We have previously reported that following VEGF treatment of endothelial cells, VEGFR-2 is phosphorylated on Tyr$^{1214}$ upstream of the Cdc42-SAPK2/p38-MAPKAP K2 pathway. However, little is known of the earliest molecular events that compose the SAPK2/p38 pathway following VEGF-2 activation. In this study, we address this question using HA-tagged constructs of either wild-type VEGFR-2 or Y1214F VEGFR-2 mutant in immunoprecipitation assays. We show that the Src family kinase member Fyn, but not c-Src itself, is recruited to VEGFR-2 and is activated in a p$^-$$^-$$^\text{Y}$$^{}$$^\text{Y}$$^{}$$^\text{Y}$$^{}$$^\text{Y}$$^{}$-dependent manner. We also report that the SH2 domain-containing adapter molecule Nck, but not Grb2, is recruited to VEGFR-2 in a p$^-$$^-$$^\text{Y}$$^{}$$^\text{Y}$$^{}$$^\text{Y}$$^{}$$^\text{Y}$$^{}$-dependent manner and that it associates with Fyn. Moreover, Pak-2 is phosphorylated in a Fyn-dependent manner. Using chemical and genetic inhibitors, we show that Fyn activity is required for SAPK2/p38 but not for FAK activation in response to VEGF. In contrast, c-Src permits activation of FAK, but not that of SAPK2/p38. In addition, Fyn is required for stress fiber formation and endothelial cell migration. We propose a model in which Fyn forms a molecular complex with Nck and Pak-2 and suggest that this complex assembles in a p$^-$$^-$$^\text{Y}$$^{}$$^\text{Y}$$^{}$$^\text{Y}$$^{}$$^\text{Y}$$^{}$-dependent manner within VEGFR-2 following VEGF treatment. In turn, this triggers the activation of the SAPK2/p38 MAP kinase module, and promotes stress fiber formation and endothelial cell migration.

Angiogenesis, the formation of blood vessels from pre-existing ones, is the major mechanism of neo-vascularization in adults. It is required in many physiological as well as pathological processes, namely embryonic development, wound healing, tissue regeneration, rheumatoid arthritis, tumor growth, and metastasis. It is regulated by a tight balance between pro- and anti-angiogenic agents, among which VEGF$^2$ is one of the most potent promoters.

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2 The abbreviations used are: VEGF, vascular endothelial growth factor; ECIS, endothelial cells growth supplement; HIVUECs, human umbilical vein endothelial cells; FAK, focal adhesion kinase; GFP, green fluorescent protein; Grb2, growth factor receptor-bound protein-2; MAP, mitogen-activated protein; HA, hemagglutinin; MAPKAP K2, MAP kinase-activated protein kinase-2; PAEC, porcine aortic endothelial cells; Pak-2, p21-activated kinase-2; PI3-K, phosphoinositide 3-kinase; PLCγ, phospholipase Cγ; SAPK2/p38, stress-activated protein kinase-2/p38; SH2, Src-homology domain-2; VEGF-2/KDR, vascular endothelial growth factor receptor-2/kinase-insert domain containing receptor; VRAP, VEGF receptor-associated protein; wt, wild type; FITC, fluorescein isothiocyanate.
recruitment of phosphoinositide 3-kinase (PI3-K) and phospholipase-Cγ (PLCγ) (10), and is important for the formation of actin stress fibers and cell migration (6). PLCγ is recruited to Tyr801 and Tyr1175 within VEGFR-2 and the corresponding Tyr799 and Tyr1169 on VEGFR-1 (11). Other studies have shown that Tyr1175 is essential for the tyrosine phosphorylation of PLCγ, MAP kinase phosphorylation, and DNA synthesis in response to VEGF-A (7). PLCγ phosphorylation also seems to depend on Y996 phosphorylation (12). Other proteins might compete for the binding to Tyr1175, like Sck (13) and Shb (14). Studies of VEGFR-1-associated proteins revealed that Tyr1213 within VEGFR-1 is a major binding site for PI3-K, Nck, and SHP-2 (15).

The Src family of protein kinases are major signaling molecules that are involved in a variety of cellular processes, namely cell proliferation, cytoskeletal alterations, differentiation, survival, adhesion, and migration (16, 17). Ten of these molecules have been identified, of which three members (Src, Fyn, and Yes) are expressed in most tissues. All Src family members comprise a N-terminal membrane localization domain (Src homology 4 or SH4 domain) that is involved in myristoylation, a poorly conserved unique domain, an SH3 and an SH2 domain involved in protein-protein interactions, a tyrosine kinase or SH1 domain, and a short C-terminal regulatory sequence (16–18). The Src family kinases have been implicated in endothelial migration or activation of SAPK2/p38. In particular, McMullen et al. (19) reported an inhibition of SAPK2/p38 by the pan Src family kinase chemical inhibitors PP1 and PP2, and an inhibition of VEGF-induced cell migration by PP1. Moreover, VEGF-mediated angiogenesis has been shown to require Src kinase activities (20). Interestingly, the recruitment of c-Src to the murine VEGFR-2 requires the phosphorylation of Tyr1212, the homologue of Tyr1214 within human VEGFR-2 (21). However, the identity of the Src family kinase that is involved in mediating VEGF-induced SAPK2/p38 in human endothelial cells is still unknown.

The aim of this study was to decipher the early molecular events required for SAPK2/p38 activation following VEGF-2 activation by VEGF. We found that Fyn, but not c-Src, is activated and recruited to a complex containing Nck-PAK-2 at the p~ Tyr1214 residue within VEGFR-2 upon VEGF treatment. We also show for the first time that activation of Fyn is required for the activation of SAPK2/p38 activation, formation of stress fibers, and endothelial cell migration.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Anti-HA clone 12Ca5 was purchased from Roche Applied Science (Laval, Québec, Canada). HA-11 monoclonal antibody was obtained from Covance (Richmond, CA). Anti-p38 is a polyclonal antibody raised in the rabbit against the C-terminal sequence PPLQEEMES of murine p38 (22). Antiphosphotyrosine clone 4G10 mouse antibody, PAK-2 rabbit antibody, and phospho-p38 MAP kinase (Thr180/Tyr182) were purchased from Cell Signaling (Beverly, MA). Src (Src 2) rabbit polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti v-Src (Ab-1) mouse monoclonal antibody was from Calbiochem (Mississauga, Ontario, Canada). Nck, Grb2, and FAK mouse antibodies were obtained from BD Biosciences (Mississauga, Ontario, Canada). Rabbit polyclonal anti-VEGF-2 (pTyr214) and rabbit polyclonal anti-Src (pTyr418) were purchased from Invitrogen (Burlington, Ontario, Canada). Monoclonal anti-p59 Fyn clone 1S, anti-FLAG M2 monoclonal, and monoclonal anti-VEGF receptor-2 (clone KDR-2) were from Sigma-Aldrich. Anti-phospho-FAK (Tyr397) was purchased from Upstate (Lake Placid, NY). Anti-GFP (living colors with peptide antibody) was purchased from ClonTech (Mountain View, CA).

**Chemicals**—PP2 and SU6656 were purchased from Calbiochem (Mississauga, Ontario, Canada). Protein A-Sepharose and Protein G-Sepharose were obtained from GE Healthcare (Piscataway, NJ). Tfx-50 was purchased from Promega (Nepean, Ontario, Canada). FITC-phalloidin, ECGS, VEGF, and Geneticin (G418) were obtained from Sigma-Aldrich.

**Plasmids**—pIRES HA-VEGF-2, pIRES Y1214F HA-VEGF-2, and pIRES Y1175F HA-VEGF-2 were generated by subcloning the previously described cDNAs (8) from pCDNA3 to pIRES hrGFP2A between Xhol and SacII restriction sites. The HA epitopes are in C-terminal. pCDNA3 HA-FAK was previously described (9). pCDNA3 Fyn wt was subcloned from pEGFP Fyn (obtained from Dr. François Marceau, Université Laval, Québec, Canada) into pCDNA3 between the HindIII and BamHI restriction sites. pCDNA3 Fyn K299M mutant was generated by site-directed mutagenesis using 5′-GGGGATCTGACTCTTTAACCAGGGTG-3′ (sense) and 5′-GGTATAGCAGTTTG-3′ (reverse). pLNCX c-Src wt and c-Src K295R were obtained from Dre. Joséé N. Lavoie (Université Laval, Québec, Canada). pEGFP C1 was purchased from BD Biosciences (Mississauga, Ontario, Canada). The expression vector pCDNA3 HA-p38 was previously described (22). pCDNA3 HA-Nckβ wt and pCDNA3 HA-Nckβ SH2 mutant (R312K) were obtained from Dr. Tom Moss (Université Laval, Québec, Canada).

**Cell Culture and Transfection**—HUVECs were isolated by collagenase digestion of umbilical veins from undamaged sections of fresh cords (22). HUVECs were grown on gelatin-coated 75 cm² culture dishes in 199 medium supplemented with 20% heat-inactivated fetal bovine serum (FBS), 60 mg of ECGS, glutamine, heparin, and antibiotics. Subcultures were obtained by trypsinization and were used at passages <5. At 16 h before experiments, HUVECs were incubated in ECGS-free medium containing 5% fetal bovine serum before addition of VEGF. PAE/VEGF-2 is a well-characterized cell line that stably expresses VEGF-2 (23). Parental PAE cells and PAE/VEGF-2 cells were grown on gelatin-coated culture dishes in F12 medium supplemented with 10% heat-inactivated fetal bovine serum and, in the case of PAE/VEGF-2 cells, with 400 µg/ml G418. For transient transfection, PAE or PAE/VEGF-2 cells were lipofected with plasmid DNAs using a ratio of 4:1 Tfx-50 (Promega) for 90 min in the absence of serum. Cells were then overlaid with complete medium and assays were carried out 24 h post-transfection. At 16 h before experiments, cells were incubated in serum-free F12 medium. NIH 3T3 clone 4F2 is a fibroblastic cell line stably expressing VEGF-2 that was previously described (8). NIH 3T3 parental cells and clone 4F2 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum, and, in
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the case of 4F2 cells, with 400 µg/ml G418. For transient expression, 1.25 × 10^6 NIH 3T3 or 4F2 cells were plated in 100-mm culture dishes and transfected with 20 µg of plasmid DNAs using the standard calcium chloride precipitation technique. At 16 h before the addition of VEGF, cells were incubated in serum-free DMEM. Assays were carried out 48-h post-transfection. All cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO₂.

Immunoprecipitation—All steps were done at 4 °C. After treatments, the cells were washed with phosphate-buffered saline and were extracted in B buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.1% sodium deoxycholate, 2 mM EDTA, 2 mM EGTA, 1 mM Na₃VO₄, 1 mM benzamide, 1 µM leupeptin, 50 mM NaF, and 1 mM phenylmethylsulfonyl fluoride. Cells were centrifuged at 16,000 × g for 10 min. Proteins were precleared for 15 min with Protein A- or Protein G-Sepharose. Supernatants were incubated on ice for 16 h with appropriate antibodies. Then, 15 µl of 50% (v/v) Protein A- or Protein G-Sepharose were added, and the incubation was extended for 60 min on ice with shaking. Antibody-antigen complexes were washed four times with B buffer and then SDS-PAGE loading buffer was added. Proteins were separated by SDS-PAGE, and the gels were transferred onto nitrocellulose for Western blotting. After incubating nitrocellulose membranes with the appropriate primary antibody, antigen-antibody complexes were detected with an anti-IgG antibody coupled to horseradish peroxidase and then were revealed using an enhanced chemiluminescence kit. Quantification of the immunoreactive bands was done by densitometric scanning using NIH Image software.

Immunofluorescence—Fluorescence microscopy was used for visualization of F-actin and GFP. HUVECs, PAEC, or PAE/VEGFR-2 cells were plated on gelatin-coated Labtek. After treatments, cells were fixed with 3.7% formaldehyde and permeabilized with 0.1% saponin in phosphate-buffered saline, pH 7.5. F-actin was detected using FITC-conjugated phalloidin (33.3 µg/ml) diluted 1:50 in phosphate buffer. Cells were examined under fluorescent microscopy with a Nikon Eclipse E600 equipped with a 40 × 0.85 NA objective lens. Images were captured as 16 bit TIFF files with a Micromax 130 YHS cooled (−30 °C) camera (Princeton Instruments, Trenton, NJ) driven by Metamorph software (Universal Imaging Corp., Downingtown, PA). Images were processed using Adobe Photoshop (Adobe Systems). At least 100 GFP-positive cells per condition were counted for quantification of stress fibers.

Cell Migration Assay—Cell migration was assayed using a modified Boyden chamber assay as described previously (24). PAE cells were co-transfected with a vector expressing GFP together with an empty vector or vectors expressing wild-type VEGFR-2 or Y1214F. Likely, PAE/KDR cells were co-transfected with a vector expressing GFP together with vectors expressing wild-type Fyn or K299M. After 48 h, the cells were made quiescent by serum starvation before being used the day after. Then, cells were harvested with trypsin, counted, centrifuged, and resuspended at 1 × 10⁶ cells/ml in migration buffer (199 medium, 10 mM HEPES, pH 7.4, 1 mM MgCl₂, 0.5% bovine serum albumin). Cells were added on the upper part of a 5.0-µm pore size polycarbonate membrane that was coated with gelatin and that separated the upper and lower chambers of a 6.5-mm Transwell apparatus. Cells were left to adhere for 1 h. Then, VEGF (5 ng/ml) was added to the lower chamber. Two hours later, cells on the upper face of the membrane were scraped using a cotton swab, and the number of fluorescent cells that had crossed the membranes was determined using an inverted fluorescence microscope. Assays were performed in triplicates.

RESULTS

Phosphorylation of Tyr1214 within VEGFR-2 Is Essential for Actin Cytoskeleton Remodeling and Endothelial Cell Migration in Response to VEGF—VEGF activates SAPK2/p38 in a VEGFR-2-dependent manner in endothelial cells, which leads to actin polymerization (25). SAPK2/p38 activation requires the phosphorylation of the major site Tyr1214 on the cytoplasmic portion of VEGFR-2 and the activity of the small GTPase Cdc42 (8). In the present study, we investigated the earliest molecular events that lead to the activation of the Cdc42-SAPK2/p38 axis downstream of VEGF-2/Tyr1214.

First, we assayed the kinetics of phosphorylation of Tyr1214 within VEGFR-2 to establish the earliest peak of phosphorylation of this site in response to VEGF. HUVECs were treated with 5 ng/ml VEGF for increasing time intervals, varying from 0.5 to 30 min. Thereafter, the phosphorylation level of VEGFR-2 was evaluated in Western blot using a phosphospecific antibody against Tyr1214 within VEGF-2. Fig. 1A shows that the VEGF-induced phosphorylation of VEGFR-2 on Tyr1214 followed a bell-shaped curve starting at 0.5 min., reaching a peak at 2 min and declining to basal level after 20 min of treatment. In accordance, VEGFR-2 is phosphorylated on Tyr1214 in VEGF-treated porcine endothelial cells (PAE), null for VEGFR-2, but in which we transiently transfected wtVEGFR-2. In contrast, VEGFR-2 is not phosphorylated on Tyr1214 in cells that express a Y1214F mutant (Fig. 1B). The rapid but transient phosphorylation is consistent with our previous findings showing that downstream targets of VEGF-2 are maximally activated by VEGF within a brief time frame that varies between 1 min (Cdc42) and 10 min (SAPK2/p38).

Phosphorylation of Tyr1214 within VEGFR-2 mediates activation of SAPK2/p38, which then plays an essential role in the regulation of the actin remodeling required for endothelial cell migration. However, the requirement of Tyr1214 within VEGFR-2 to initiate actin remodeling and cell migration has never been shown directly. We therefore looked at the VEGF-induced actin phenotype in PAE cells that have a null background for VEGFR-2 and that were co-transfected to transiently express either wild-type VEGFR-2 or the phosphorylation mutants Y1214F or Y1175F, along with GFP. We found that untransfected PAE cells, or cells transfected with an empty vector (Fig. 1C, panels a and b) exhibit a basal level of transcytoplastmic stress fibers that was markedly increased by VEGF in PAE cells expressing the wild-type VEGFR-2 or the Y1175F mutant (with 44% and 39% of transfected cells displaying stress fibers, respectively; see arrows in Fig. 1C, panels c and d and g and h, and Fig. 1D). In contrast, the cells that expressed the Y1214F mutant lacked transcytoplastmic stress fibers (with only 5% of transfected cells displaying stress fibers; see arrows in Fig.
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A

| VEGF (min) | 0.5 | 1 | 2 | 5 | 10 | 20 | 30 |
|------------|-----|---|---|---|----|----|----|
| WB P~1214  |     |   |   |   |    |    |    |
| Fold activation | 1.0 | 2.6 | 2.7 | 4.8 | 3.4 | 3.0 | 1.3 | 0.3 |
| WB VEGFR-2 |     |   |   |   |    |    |    |

B

C

Empty vector  VEGFR-2 wt  Y1214F  Y1175F

Actin

GFP

VEGF 5ng/ml 15 min

D

| VEGF | Empty vector | VEGFR-2 wt | Y1214F | Y1175F |
|------|--------------|------------|--------|--------|
|      |              |            |        |        |
| -    |              |            |        |        |
| +    |              |            |        |        |

Percentage of GFP-positive cells displaying stress fibers

E

| VEGF | Empty vector | VEGFR-2 wt | Y1214F |
|------|--------------|------------|--------|
| -    |              |            |        |
| +    |              |            |        |

Number of migrating cells

WB HA

VEGFR-2 wt  Y1214F
1C, panels e and f (and Fig. 1D). The reorganization of the actin cytoskeleton into stress fibers is thought to generate the tension within the cells that is required for cell migration. Considering the role of VEGFR-2 and SAPK2/p38 in this process, we investigated the role of the VEGFR-2 Tyr\textsuperscript{1214} in migration assays using Boyden chambers. PAE cells were transiently co-transfected with GFP and either wild-type VEGFR-2 or Y1214F mutant. Cells seeded in the upper chamber were subject to a VEGF chemoattractive treatment in the lower chamber. As shown in Fig. 1E, the cells expressing the Y1214F mutant displayed a significantly reduced migratory response compared with cells expressing wtVEGFR-2. These results are the first direct evidence indicating that phosphorylation of Tyr\textsuperscript{1214} within VEGFR-2 is required to trigger VEGF-induced actin remodeling and endothelial cell migration, which is consistent with its role in activating SAPK2/p38. Of note, the expression of Y1214F did not totally inhibit cell migration. This suggests that other migratory pathways are activated downstream of other Tyr residues within VEGFR-2. In this context, the activation of PI3-K downstream of Tyr\textsuperscript{1175} also regulates endothelial cell migration by VEGF (14). Recently, it was shown that recruitment of the TSAd/VRAP adaptor to p\textsuperscript{−}Tyr\textsuperscript{951} of VEGFR-2 also has a crucial effect on endothelial cell migration (6).

**Nck Is Recruited to VEGFR-2 in a p\textsuperscript{−}Tyr\textsuperscript{1214}-dependent Manner**—A number of adapter molecules are known to associate with activated VEGFR-2. These molecules bind to phosphorylated tyrosine residues via their SH2 domain leading to activation of several intracellular signaling pathways. Using immunoprecipitation assays, we investigated which adapter molecules are recruited to phosphorylated Tyr\textsuperscript{1214} upon VEGF treatment. We first sort out the published consensus sequences of the SH2 domain of a number of candidate adapter proteins that could be recruited to the amino acid environment of Tyr\textsuperscript{1214}. In addition to c-Src (26), two adapter proteins, Grb2 (27) and Nck (28), were found to possess an SH2 domain compatible with the recruitment to Tyr\textsuperscript{1214} (Table 1). We next examined whether these proteins were indeed recruited to Tyr\textsuperscript{1214}.

NHI-3T3 cells were transfected with plasmids expressing GFP together with the wild-type form of HA-VEGFR-2 or the mutant variant Y1214F of HA-VEGFR-2. They were then treated or not with VEGF. Thereafter, extracts were prepared and Grb2 or Nck were detected with the immunoprecipitated receptors. Results showed that Grb2 was rapidly recruited to VEGFR-2 in response to VEGF. However, its recruitment to VEGFR-2 proceeded through a Tyr\textsuperscript{1214}-independent manner, being not inhibited in cells expressing the Y1214F mutant (Fig. 2A). In contrast, we found that Nck co-immunoprecipitated with activated VEGFR-2, and that its recruitment was inhibited by the expression of the Y1214F HA-VEGFR-2 mutant (Fig. 2B). Moreover, Nck was phosphorylated on tyrosine in a Tyr\textsuperscript{1214}-dependent manner following VEGF (Fig. 3A). These results suggest that Nck is an adapter that is recruited to Tyr\textsuperscript{1214} of VEGFR-2 and therefore suggests its involvement in activating SAPK2/p38. Accordingly, the transfection of an Nck construct containing a point mutation (R312K) in its SH2 domain was not recruited to VEGF-2 and was associated with an inhibition of the VEGF-induced SAPK2/p38 activation (Fig. 3, B and C). Overexpression of the wild-type form of Nckβ did not enhance SAPK2/p38 activation in absence of VEGF, which is in accordance with its role as an adapter molecule devoid of intrinsic catalytic activity. Altogether, these results indicate that Nck is an adapter protein that connects Tyr\textsuperscript{1214} within VEGFR-2 to the activation of SAPK2/p38.

**Fyn Is Recruited to VEGFR-2 and Activated in a Tyr\textsuperscript{1214} Phosphorylation-dependent Manner**—The Src family kinases have been implicated in VEGF signaling and SAPK2/p38 activation (26). Moreover, the amino acid environment of Tyr\textsuperscript{1214} displays similarity with the binding site of c-Src SH2 domain (Table 1). We thus investigated whether Src kinases were involved in transducing the VEGF signal to SAPK2/p38. Immunoprecipitation of HA-VEGFR-2 or Y1214F HA-VEGFR-2 constructs were used in transfection studies performed in NIH-3T3 cells to ascertain the recruitment of Src family kinases. By using Src2, a rabbit polyclonal antibody thatrecognizes several members of the Src family kinases, we found that at least one Src kinase was recruited in a Tyr\textsuperscript{1214}-dependent manner to activated VEGFR-2, as previously reported (Fig. 4A and Ref. 26). However, specific detection of c-Src by means of two different specific antibodies revealed that c-Src was not recruited to VEGFR-2 within the first 5 min of exposure to VEGF (Fig. 4B, 2A).
left panel and data not shown). Yet, c-Src could be immuno-precipitated with FAK in response to VEGF, which demonstrates the reliability of the anti-c-Src antibodies (Fig. 4B, right panel and data not shown). Interestingly, c-Src was markedly phosphorylated within 1 min on its autophosphorylation site Tyr\(^{418}\) indicating that it was activated in response to VEGF (Fig. 4C). Consistent with the fact that c-Src was not recruited to VEGF-2, its activation was independent of Tyr\(^{1214}\) phosphorylation (Fig. 4C). The phosphorylation of c-Src on Tyr\(^{418}\) was transient and returned to near basal level after 5 min (Fig. 4C). Intriguingly, c-Src was still activated after 5 min of VEGF treatment in Y1214F-expressing cells, suggesting that a phosphatase might be recruited to VEGF-2 in a p–Tyr\(^{1214}\)-dependent manner and thus plays a role in c-Src dephosphorylation. It was previously reported that the phosphatase SHP-2 is recruited to Tyr\(^{1215}\) of VEGF-1 (15). Therefore, mutation of the Tyr\(^{1214}\) residue within VEGF-2 could maintain the VEGF-induced activation of c-Src, by delaying its dephosphorylation. Such a possibility remains to be ascertained. In contrast, Fyn was activated in response to VEGF, but this activation was inhibited in cells expressing the Y1214F mutant indicating that it requires the phosphorylation of Tyr\(^{1214}\) (Fig. 4D). In accordance, Fyn was recruited to VEGF-2, and its recruitment occurred in a Tyr\(^{1214}\)-dependent manner, despite the absence of a consensus recognition sequence for the SH2 domain of Fyn in the amino acid environment of Tyr\(^{1214}\) (Fig. 4E, Table 1 and Refs. 29 and 30). This strongly suggested that the Tyr\(^{1214}\)-mediated recruitment of Fyn was indirect and did not rely on a direct interaction between Fyn and Tyr\(^{1214}\).
Overall, these results suggest that the ubiquitously expressed Src family kinase member Fyn could play a role in the activation of downstream targets following VEGFR-2 activation.

Activation of Fyn Is Required for VEGF-induced Activation of SAPK2/p38—To investigate the role of Fyn on SAPK2/p38 activation, we first pretreated HUVECs with the pan Src family kinases inhibitors PP2 or SU6656 and then treated the cells with VEGF. Consistent with a role of Fyn in the SAPK2/p38 pathway, we found that both PP2 and SU6656 impair SAPK2/p38 activation in response to VEGF in endothelial cells (Fig. 5A). To further ascertain the role of Fyn in the activation of SAPK2/p38, we transiently expressed a wt form of Fyn or the K299M mutant, a kinase-dead mutant of Fyn in 4F2 cells, an NIH 3T3 derived-clonal cell line that stably expresses VEGFR-2 (8). Then, we verified the activation of SAPK2/p38 in response to VEGF using phosphospecific antibodies. We found that the activation of p38 by VEGF was markedly enhanced in cells expressing the wild-type form of Fyn but that the Fyn K299M mutant impaired the VEGF-induced activation of SAPK2/p38 (Fig. 5B). However, the expression of the mutant did not inhibit the VEGF-induced activation of FAK (data not shown). The mutation K299M on Fyn is located in the catalytic domain and affects its activation, as shown in Fig. 5C (31). In contrast, a c-Src kinase-dead mutant K295R did not impair SAPK2/p38 activation, yet it inhibited FAK phosphorylation on the autophosphorylation site Tyr397 in response to VEGF (Fig. 5D and data not shown). These results suggest that Fyn is implicated in conveying the VEGF signal to SAPK2/p38, while c-Src is required for FAK activation in response to VEGF.

Activation of Fyn Is Required for VEGF-induced Actin Remodeling and Endothelial Cell Migration—To further highlight the functions of Tyr_1214 within VEGFR-2 as well as...
of Fyn in VEGF signaling, we transiently expressed a wild-type or a dominant negative form of Fyn in PAE/VEGFR-2 cells and looked at the actin phenotype in response to VEGF. We found that the expression of wt Fyn enhances the formation of stress fibers in response to VEGF compared with cells transfected with an empty vector but that the Fyn K299M mutant inhibited this actin reorganization (Fig. 6A). 38% cells transfected with wild-type Fyn displayed stress fibers, compared with 5% in the Fyn K299M mutant transfected cells (Fig. 6B). Moreover, the expression of wild-type Fyn triggers VEGF-induced endothelial cell migration whereas the expression of the Fyn K299M mutant inhibited the process (Fig. 6C). Altogether, these results suggest that Fyn is activated by VEGF downstream of Tyr1214, which leads to SAPK2/p38-dependent actin reorganization into stress fibers and endothelial cell migration.

**Fyn Associates with Nck in Response to VEGF**—As previously stated, the VEGFR-2 amino acid sequence does not possess any known consensus binding site for the SH2 domain of Fyn (see Table 1). Because the phosphorylation of Fyn depends on the phosphorylation state of VEGFR-2 on Tyr1214 (Fig. 4D), this suggested that Fyn could be recruited on VEGFR-2 in an indirect manner. Given that our results are consistent with the fact that Nck is recruited to Tyr1214, we thus verified whether Fyn associates with Nck in response to VEGF. We found that Fyn and Nck do indeed associate because they can be co-immunoprecipitated following VEGF treatment. However, the association of Nck to Fyn was impaired by the K299M mutation (Fig. 7A). Moreover, we found that Fyn but not the Fyn kinase-dead mutant can phosphorylate Nck in the presence of VEGF (Fig. 7B). This suggests that the tight association of Fyn and Nck might depend on a Fyn-dependent phosphorylation of Nck. Interestingly, we further found that another protein of ~65kDa that co-immunoprecipitated with Nck was also tyrosine-phosphorylated in a Fyn-dependent manner by VEGF. Because the 65-kDa p21-activated kinases (PAK) are known to associate with Nck and could be involved in SAPK2/p38 activation (32–34), we investigated whether PAK was phosphorylated in a Fyn-dependent manner by VEGF. In line with this, we found that the ubiquitous kinase PAK-2 was phosphorylated on tyrosine in response to VEGF in cells that express VEGFR-2 along with wild-type Fyn but not with the K299M mutant (Fig. 7C). Consistent with the fact that Fyn activation depends on the p-Tyr1214 residue, the phosphorylation of PAK-2 remains near basal level in cells expressing wild-type Fyn and the Y1214F mutant (Fig. 7C). The slight phosphorylation of PAK-2 that is still detectable in HA-1214F/Fyn-transfected cells is imputable to the intrinsic basal activity of Fyn when overexpressed (see Fig. 4D and Ref. 35). These data suggest a possible role for PAK-2 in SAPK2/p38 activation. Of note, PAK-1 was undetectable in NIH 3T3 cells (data not shown). Altogether, these data suggest that Fyn is recruited to Nck on Tyr1214 within VEGFR-2. Fyn will then contribute to initiate a cascade of phosphorylation events involving Nck and PAK-2 and leading to SAPK2/p38 activation and to actin remodeling and cell migration.

**DISCUSSION**

VEGF binds to the tyrosine kinase receptor VEGFR-2 in endothelial cells, which activates different signaling pathways that regulate the biological functions of VEGF. The activation of these pathways originates from the phosphoryl-
ation of precise tyrosine residues on the cytoplasmic portion of VEGFR-2. Takahashi et al. (7) have identified Tyr1175 and Tyr1214 as the major autophosphorylation sites on VEGFR-2. The phosphorylated Tyr1175 residue conveys the VEGF signal to the MAP kinase ERK, via the phosphorylation of PLCγ and activation of the Ras-Raf-MEK1 pathway (7).

In vivo, homozygous knocking-in mice in which the corresponding Tyr1173 is substituted for Y1173F show severe defects in blood vessels organization indicating that the phosphorylation of this site is essential for normal vascularization during embryogenesis (36). In the case of Tyr1214, we have previously shown that its phosphorylation is required for the activation of the Cdc42-SAPK2/p38 pathway and to regulate the reorganization of the actin cytoskeleton into stress fibers (8, 25). Intriguingly, the knock-in homozygous Y1212F mice remain viable and fertile suggesting that the major role of its homologue Tyr1214 in human is to regulate angiogenesis in the adult rather than during embryogenesis (36).

In the present study, we brought further insights in deciphering the VEGFR-2-SAPK2/p38 pathway. Our major contribution is to have characterized some of the earliest events that connect VEGFR-2 to SAPK2/p38-mediated actin remodeling and endothelial cell migration. In particular, we found that Tyr1214 within VEGFR-2 is quickly phosphorylated in response to VEGF reaching a peak of phosphorylation within 2 min. This triggers the recruitment to VEGFR-2 of the adapter protein Nck and of the Src kinase family member Fyn. In turn, Fyn is activated, which is associated with the phosphorylation of Nck
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**Figure 7.** Fyn activity is required for Nck association and for phosphorylation of PAK-2 in response to VEGF. A, NIH 3T3 cells were plated on 100-mm Petri dishes and transfected with GFP, or co-transfected with wild-type VEGFR-2 and wild-type Fyn or K299M Fyn mutant for 48 h. Cells were serum-starved for 16 h, treated with 10 ng/ml VEGF for the indicated periods of times and then extracted with B Buffer. Fyn was immunoprecipitated and the whole samples were loaded on SDS-PAGE, transferred to nitrocellulose and blotted with Nck antibody. Total Nck and HA-tagged receptors were used to ensure equal protein loading and VEGFR-2 expression. B, NIH 3T3 cells were transfected as in A. Nck was immunoprecipitated, and the whole samples were loaded on SDS-PAGE, transferred to nitrocellulose and blotted with phosphotyrosine 4G10 antibody. Total Nck, Fyn, and HA-tagged receptors were used to ensure equal protein loading and expression. C, NIH 3T3 cells were transfected with GFP or co-transfected with either VEGFR-2 or Y1214F mutant and wild-type Fyn or K299M Fyn mutant, as indicated. PAK-2 was immunoprecipitated, and the whole samples were loaded on SDS-PAGE, transferred to nitrocellulose, and blotted with phosphotyrosine 4G10 antibody. The membrane was re-blotted for PAK-2. Total Fyn- and HA-tagged receptors were used to ensure equal protein loading and expression. Experiments were repeated at least twice with similar results.

Our demonstration that Tyr$^{1214}$ is maximally phosphorylated within 2 min of exposure to VEGF is the first report showing the quick responsiveness of this site to activation of VEGFR-2. This finding is consistent with the fact that Tyr$^{1214}$ is an auto-phosphorylation site within VEGFR-2 and with previous studies reporting the VEGF-induced quick activation of the autokinase activity and phosphorylation of VEGFR-2 (7, 37). On the other hand, the fact that the phosphorylation of Tyr$^{1214}$ is transient and declines to basal level after 20 min is also new and suggests that p-Tyr$^{1214}$ is quickly dephosphorylated by a phosphatase. In this context, tyrosine phosphatases SHP2 and PTP1b are rapidly recruited to activated VEGFR-2 suggesting that they might be involved in dephosphorylating p-Tyr$^{1214}$ (38, 39). We recently found that VEGFR-2 is internalized after 30 min of activation, which reached the possibility that the dephosphorylation of p-Tyr$^{1214}$ may occur during the internalization process.3 Most interestingly, our finding that Tyr$^{1214}$ is maximally phosphorylated after 2 min of exposure to VEGF is consistent with our previous study showing that the sequential activation of Cdc42 and SAPK/p38 downstream of Tyr$^{1214}$ are maximal within 1 min and 10 min, respectively (8, 25). Moreover, another important issue of our study is to bring the first direct evidence that phosphorylation of Tyr$^{1214}$ is required for cell migration since the expression of Y1214F is associated with an inhibition of VEGF-induced cell migration. The direct demonstration that phosphorylation of Tyr$^{1214}$ is essential for cell migration is in line with our previous finding that this event is connected to SAPK2/p38-mediated activation of cell migration. The finding that the expression of Y1214F did not totally inhibit cell migration is interesting and suggests the participation of other migratory pathways that are activated downstream of other Tyr residues within VEGFR-2. In this context, it has been reported that the activation of PI3-K downstream of Tyr$^{1770}$ also regulates endothelial cell migration by VEGF (14). Also, the adapter TSAd/VRAP has been shown to bind to the Tyr$^{951}$ residue within VEGFR-2 and regulates cell migration (15). Therefore, Tyr$^{1214}$ appears to be essential but not sufficient alone for the regulation of endothelial cell migration. Otherwise, our finding that phosphorylation of Tyr$^{1214}$ is essential to the formation of stress fibers is a further indication that these actin structures are an essential step of cell migration, presumably by contributing to confer the traction forces required to draw the rear of the moving cells toward the leading edge.

An important issue in the biology of cell signaling is to identify the earliest molecular events associated with receptor activation since the identification of these molecules may provide structural features to understand how the spatial alignment of the signaling proteins of a given pathway ensures efficient signaling. In addition, the identification of these early signaling events will allow developing drugs enabling to shut-off or enhancing a given signal from its inception.

In this context, we made significant progress in understanding the earliest VEGF signals that emanate from Tyr$^{1214}$ within VEGFR-2. Our results show that, within 1 min of VEGF treatment, the SH2-containing adapter molecule Nck is recruited to VEGFR-2 and is tyrosine-phosphorylated in a Tyr$^{1214}$-dependent manner. Interestingly, the amino acid environment of Tyr$^{1214}$ displays similarity with the consensus recognition sequence of the SH2 domain of Nck, which suggests that Nck might be directly recruited to the Tyr$^{1214}$ within VEGFR-2. This is further supported by our finding that the expression of Nckβ containing a point mutation in its SH2 domain is associ-
ated with an inhibition of SAPK2/p38, the activation of which is tightly associated with phosphorylation of VEGF-2 on Tyr\textsuperscript{1214} in response to VEGF (8). However, one cannot exclude the possibility that the recruitment of Nck to Tyr\textsuperscript{1214} is indirect and requires an earlier intermediate such as FRS2, as previously suggested (34). Intriguingly, the adapter molecule Grb2 also possesses a suitable environment for binding to Tyr\textsuperscript{1214}. However, immunoprecipitation of HA-tagged VEGF-2 constructs showed that Grb2 recruitment is independent of Tyr\textsuperscript{1214} phosphorylation. This means that Grb2 is recruited on another tyrosine residue on VEGF-2. Recruitment of Grb2 to VEGF-2 has previously been reported in porcine endothelial cells (40), but this association was not detected in primary cultures of sinusoidal endothelial cells in response to VEGF (41). Hence, the status of Grb2 in VEGF-2 signaling remains to be defined. However, because the association of Grb2 to a tyrosine kinase receptor may activate the Ras-Raf1-MEK-ERK pathway, it is likely that Grb2 plays a role in VEGF signaling (5).

Nck possesses three SH3 domains that interact with at least 35 different molecules, some of which play important roles in actin reorganization. In particular, PAK can bind the second SH3 domain of Nck through its proline-rich sequence in the N terminus. In response to VEGF, the Nck-PAK complex is recruited to VEGF-2, and both Nck and PAK are phosphorylated on tyrosine during the process. In turn, this is followed by an increase in endothelial cell migration through regulation of actin polymerization and focal adhesion turnover (33, 34). However, the kinase involved in phosphorylating Nck or PAK is unknown. The major contribution of our study is to provide evidence indicating that the Src family kinase Fyn is activated in a Tyr\textsuperscript{1214}-dependent manner, which triggers the phosphorylation of Nck-PAK-2 and then of SAPK2/p38 leading to actin remodeling and cell migration.

In particular, we found that Fyn is recruited within 1 min to VEGF-2 and that the recruitment is p-Tyr\textsuperscript{1214}-dependent since it is impaired in cells expressing the Y1214F mutant. By using similar experimental approach, namely transfection of wild-type VEGF-2 versus Y1214F mutant VEGF-2, we found that Fyn is phosphorylated and activated in a p-Tyr\textsuperscript{1214}-dependent manner in response to VEGF. Most importantly, our findings that Fyn activation is required to trigger activation of SAPK2/p38, actin remodeling and cell migration is supported by our results showing that these processes are abrogated in cell expressing a kinase-dead version of Fyn. These finding are in line with a recent study showing that Fyn kinase acts upstream of p38 to enable mast cells migration toward stem cell factor (42). In contrast, c-Src is not recruited to VEGF-2, at least within the first 5 min of treatment, even if it is activated by VEGF. However, c-Src does associate with FAK in response to VEGF. These results are consistent with our previous findings indicating that c-Src transduces the VEGF signal to FAK through the activation of integrin \(\alpha\)v\(\beta\)3 associated with VEGF-2 rather than through VEGF-2 itself (9).

We further found that Fyn and Nck associate in a p-Tyr\textsuperscript{1214}-dependent manner in response to activation of VEGF-2. The association of Fyn and Nck is dependent on Fyn activity being inhibited by a kinase-dead version of Fyn. These findings are consistent with the fact that the amino acid environment of Tyr\textsuperscript{1214} is more closely related to the recognition sequence of Nck-SH2 than of Fyn-SH2. Most importantly, we also found that Nck and its known partner PAK-2 are tyrosine-phosphorylated in a manner that depends on the increased Fyn activity that is induced by VEGF. This finding is important since it may provide the link that connects VEGF-2/Fyn to Cdc42/SAPK2/p38. Indeed, it has been reported that PAK-2 may be selective for Cdc42 and that Cdc42-mediated activation primes PAK-2 for superactivation by tyrosine phosphorylation (43, 44). Given that PAK-2 is a well known intermediate between Cdc42 and SAPK2/p38 one may propose that the activation of the latter downstream of Tyr\textsuperscript{1214} results from both an activation of Cdc42.
as previously reported (8) as well as from a Fyn-mediated superactivation of PK-2. Moreover, it is possible that the activation of Cdc42 may rely on Fyn activity as previously reported (31). These possibilities are in accordance with our finding that the kinase-dead version of Fyn abolishes the VEGFR-2-mediated activation of SAPK2/p38. Further studies should be undertaken to identify the precise mechanism that leads to activation of Cdc42 and PAK-2 by Fyn. On the other hand, it has been reported that the Nck:PAK-2 complex modulates actin dynamics by regulating focal adhesion turnover and WASP-mediated actin polymerization (33, 34). Our finding that the Nck:Fyn complex comprising PAK-2 is a component of the SAPK2/p38 pathway mediating actin remodeling in response to VEGF highlights a new mechanism by which these proteins modulate cytoskeletal dynamics.

Most of the molecular events reported herein take place within a minute on Tyr2124 within VEGFR-2 (Nck recruitment and tyrosine phosphorylation, Fyn recruitment and activation, Nck and Fyn interaction, PAK-2 tyrosine phosphorylation). Hence, it is difficult to establish a precise sequence of events based on time course alone. Nevertheless, our data indicate that a molecular complex is quickly assembled at VEGFR-2 and that it may follow a sequence of events that derive initially from VEGF binding to VEGFR-2 and then from the autophosphorylation of Tyr2124. In turn, this would trigger the recruitment of an Nck:PAK-2 complex to p-Tyr2124 as well as that of Fyn on Nck. Activated Fyn will then initiate the phosphorylation of Nck and that of PAK-2. This might contribute to PAK-2 activation downstream of Cdc42 and lead to the activation of the SAPK2/p38 MAP kinase module MKK3-p38-MAPKAPK2 that regulates actin reorganization and stress fiber formation. These actin structures will generate a contractile force within the cell to allow endothelial cell migration (Fig. 8, working model).

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