Vascular Endothelial Growth Factor Causes Translocation of p47^{phox} to Membrane Ruffles through WAVE1*

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Growth factors initiate cytoskeletal rearrangements tightly coordinated with nuclear signaling events. We hypothesized that the angiogenic growth factor, vascular endothelial growth factor (VEGF), may utilize oxidants that are site-directed to a complex critical to both cytoskeletal and mitogenic signaling. We identified the WASP-family verprolin homologous protein-1 (WAVE1) as a binding partner for the NADPH oxidase adapter p47^{phox} within membrane ruffles of VEGF-stimulated cells. Within 15 min of VEGF stimulation, p47^{phox} coprecipitated with WAVE1, with the ruffle and oxidase agonist Rac1, and with the Rac1 effector PAK1. VEGF also increased p47^{phox} phosphorylation, oxidant production, and ruffle formation, all of which were dependent upon PAK1 kinase activity. The antioxidant Mn (III) tetraakis(4-benzoic acid) porphyrin decreased c-Jun N-terminal kinase (JNK) activation. However, the relationship between these two processes and the signal pathways leading to these events have not been clearly defined.

Reactive oxidants have been linked to both proliferative pathways and cytoskeletal dynamics and thus may present a common mediator of these two facets of growth factor signaling. PDGF, epidermal growth factor, and insulin initiate a small burst of oxidants critical for protein tyrosine phosphorylation, MAPK activation, and DNA synthesis, whereas oxidant scavengers suppress these events (1–3). Rac1, through its involvement with the NADPH oxidase, also mediates H-RasV12-induced mitogenesis and PDGF-dependent cyclin D1 expression (4, 5). The use of oxidants is not limited to pathways linked to tyrosine kinase receptors, because smooth muscle NADPH oxidase is activated during the proliferative signaling of both angiotensin II and the ganglioside GD3 (6, 7).

Oxidants have also been linked to cytoskeletal reorganization. High levels of exogenous oxidants increase recruitment of actin to the cytoskeleton, and conversely actin cytoskeletal disruption precipitates oxidant-dependent NF-κB activation (8, 9). Oxidant production is also most prominent in actively migrating endothelial cells at the margin of a monolayer wound and, in particular, is concentrated in membrane ruffles of these highly motile cells (10). This localization of oxidant production is comparable to that found in phosphol-ester-stimulated neutrophils whose oxidative burst is concentrated within ruffling membranes (11). Consistent with these observations, we recently found constitutive localization of the NADPH oxidase subunit p47^{phox} to the cytoskeleton of endothelial cells with translocation of p47-GFP to membrane ruffles (12).

In the present study, we identify the ruffle catalyst WAVE1/ suppressor of cAMP receptors as a binding partner of p47^{phox}. Upon stimulation of endothelial cells with VEGF, p47^{phox} associates with WAVE1 as well as with Rac1 and PAK1, proteins involved in both ruffle formation and oxidase activation. p47^{phox}-dependent oxidants appear to mediate VEGF-induced ruffle formation, tyrosine phosphorylation, and JNK activation.

Following stimulation with growth factors, parenchymal cells enter a proliferative state and migrate directionally. Accordingly, growth factors initiate both mitogenic signaling through the MAPKs as well as actin cytoskeletal rearrangements associated with migration, as membrane ruffling. However, the relationship between these two processes and the

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The abbreviations used are: MAPK, mitogen-activated protein kinase; PDGF, platelet-derived growth factor; GFP, green fluorescent protein; JNK, c-Jun N-terminal kinase; HUVEC, human umbilical vein endothelial cell; HA, hemagglutinin; EGFP, enhanced GFP; GST, glutathione S-transferase; Ad, adenovirus; VEGF, vascular endothelial growth factor; DCF, dichlorofluorescein diacetate; SH, Src homology; PX, Phox; DN, dominant-negative; WASP, Wiskott-Aldrich syndrome protein; PAK, p21-activated kinase; BD, binding domain; DaRad, Discoma sp. Red; SOD, superoxide dismutase.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Full-length WAVE1 was PCR-amplified from a HUVEC library between exogenous EcoRI and Sall sites and captured in pCR4-Blunt-TOPO (Invitrogen) and then subcloned into pCI-neo (pCIN-WAVE1). Full-length WAVE1, WAVE1-(1–442), WAVE1-(1–362), and WAVE1-(1–269) were amplified from pCIN-WAVE1 with the addition of terminal EcoRI and BamHI sites and ligated into the yeast shuttle vector pGADT7. All other WAVE1 truncations were similarly amplified from the original library clone retrieved from the two-hybrid screen and ligated into pGADT7. pCIN-HA was produced by the insertion of a 3× HA tag upstream of the EcoRI site of pCI-neo. WAVE1-(270–559), WAVE1-(1–269), and WAVE1-(100–442) were then subcloned into pCI-neo with frame correction. WAVE1-(270–442) and p47-(153–286) were PCR-amplified with terminal EcoRI and XhoI sites and ligated into pGADT7. pCIN-HA was produced by the insertion of a 3× HA tag upstream of the EcoRI site of pCI-neo.
FIG. 1. Interaction of WAVE1 and p47phox. a, [35S]methionine-labeled full-length WAVE1 bound GST-p47-(1–298) but not GST-p47 full-length (FL), GST, or GSH-Sepharose beads. Top panels show Coomassie Blue stain of GST fusion inputs (right) and [35S]methionine WAVE1 translation reaction input. b, domain mapping of the WAVE1-binding p47phox region. Yeast-mating studies were performed using Gal4-AD-WAVE1 with various truncations of Gal4-BD-p47 using lacZ expression as a readout. Avid binding of WAVE1 to the tandem SH3ab domains was demonstrated. Interaction of WAVE1 with the N-terminal SH3a domain led to greater lacZ expression than with the C-terminal SH3b domain. An SH3-disrupting W193R mutation had no effect on WAVE1 binding to the SH3a domain of p47phox. The PX, proline-rich (PR), and arginine-rich (AR) domains were unnecessary for WAVE1 binding. c, domain mapping of the p47phox binding region of WAVE1. Yeast-mating studies were again performed, in this case, testing truncations of Gal4-AD-WAVE1 against Gal4-BD-p47-(1–298). Suppressor of cAMP receptor homology (SHD), basic (B), PR, verprolin-homologous (V), and acidic (A) domains are shown. d, FL or various truncations of WAVE1 were translated in vitro and bound to GST-p47-(1–298). Binding of WAVE1 to GST alone is shown as a negative control (left lane). Translation inputs are shown in top panel, and bound proteins are shown in bottom panel. GST fusion protein input is shown in a.
the addition of terminal EcoRI sites to subclone into pGEX-2TK. pGEX-p47-(full-length) was described previously (13). WAVE1 was fused to the N terminus of EGFP through amplification of pCIN-WAVE1 with the addition of terminal EcoRI and BamHI sites and removal of the endogenous stop codon and ligation into pEGFP-N3 (Clontech). p47phox was fused to the C terminus of DsRed by ligation of full-length p47phox into the EcoRI site of pDsRed2-C1 (Clontech). HA-JNK1, HA-JNK2, p47-GFP, and pCINF-p47 were previously described (12, 13). pCINF-WAVE1-(100–442) was derived by amplification of the WAVE1 fragment between exogenous EcoRI and SalI sites and ligation into pCINF. The HA-tagged APF mutants of human JNK1 and JNK2 were a gift of Dr. Lynn Heasley. Actin-GFP was obtained from Clontech. Myc-PAK1(K298A) was a gift of Dr. Melanie Cobb. All of the PCR reactions were performed with either Pfu Turbo (Stratagene) or Pfx Platinum (Invitrogen), and all of the constructs were confirmed by sequencing.

Yeast Two-hybrid Screening and Mating—AH109 yeast (Clontech)
Transfection was performed after cell synchronization with growth factor, ascorbic acid, hydrocortisone, heparin, and gentamicin.

Epidermal growth factor, fibroblast growth factor, VEGF, insulin were propagated in EGM-2 medium containing 2% fetal calf serum, equal m.o.i. (S303D, S304D, S328D) proteins were equivalent by immunoblot at 95%. Expression of p47-wild type, p47-(W193R), and p47**(1–298)** transfected HUVEC were stimulated with VEGF for 15 min and immunoprecipitated with either irrelevant antibody or mouse anti-p47**phox** and probed with antibodies against WAVE1 (Upstate Biotechnology), p47**(phox)** (Upstate Biotechnology). Immunoblots were performed with antibodies against WAVE1, p47**(phox)**, HUVEC (Clonetics) used in this study.

Cell Culture and Microscopy—HUVEC (Clonetics) used in this study were stably transfected with pGBKTT7-p47**(1–298)** and tested negative for autonomous transactivation. A HUVEC library previously dropped from pGAL4-AD (13) was used to secondarily transform bait-containing yeast, which were selected under high stringency for bait and library vectors (reversal of Trp and Leu auxotrophy) and interaction (reversal of His and Ade auxotrophy and lacZ expression). Surviving colonies were recloned and retested, and library plasmids were extracted, passaged through Escherichia coli, characterized, and transformed into AH109 yeast. Library plasmid-containing AH109 yeast were mated with bait-containing Y187 yeast, and diploids were plated and assessed for lacZ expression with a filter lift assay. Truncated forms of p47**phox** and WAVE1 were tested for interaction using a similar yeast mating strategy. Strongly positive interactions caused blue color development within 2 h. Negative interactions remained white for >24 h. Weak positive interactions caused distinct blue coloration between 2 and 24 h.

**GST Pull Down Assay—**BL21-RP E. coli (Stratagene) harboring pGEX-2TK, pGEX-p47, or pGEX-p47**(1–289)** were induced with isopropyl-1-thio-β-D-galactopyranoside for 4 h, and the soluble proteins were captured on glutathione-Sepharose 4B (Amersham Biosciences). Full-length or truncated WAVE1 proteins were transcribed and translated in vitro from pCIN-WAVE1 in the presence of [35S]methionine using the TNT Quick Coupled system (Promega). 5 µg of the fusion proteins were incubated with 10 µl of translation mixture for 2 h at 4°C prior to extensive washing.

Adenoviral Construction—Adenoviruses harboring lacZ and p47**phox** were described previously (12, 13). Sequential site-directed mutations were introduced with PCR to create p47**(S303D,S304D,S328D)**, which was then subcloned into pShuttle and then pAdX (Clontech). Ad-p47**(W193R)** and Ad-p47**(S303D,S304D,S328D)** were generated, cloned, and characterized in human embryonic kidney 293 cells. Efficiency of transgene expression in HUVEC was assessed by lacZ expression was >95%. Expression of p47-wild type, p47**(W193R)**, and p47**(S303D,S304D,S328D)** proteins were equivalent by immunoblot at equal m.o.i.

Cell Culture and Microscopy—HUVEC (Clonetics) used in this study were propagated in EGM-2 medium containing 2% fetal calf serum, epidermal growth factor, fibroblast growth factor, VEGF, insulin growth factor, ascorbic acid, hydrocortisone, heparin, and gentamicin. Transfection was performed after cell synchronization 6–8 h after thymidine release using electroporation with a constant amount of total plasmid DNA. For adenoviral transduction, cells were infected for 1 h at an m.o.i. of 200:1, and harvested 24 h later. For microscopy experiments, cells were plated on fibronectin-coated chambered glass coverslips and examined live. Prior to VEGF stimulation, cells were serum-starved in medium containing 0.1% fetal calf serum without growth factors for 24 h. For some experiments, cells were treated with 100 µM SOD mimetic MnTBAP (Calbiochem) for 1 h at 37°C. The number of ruffling cells was counted in all of the actin-GFP-expressing cells in at least ten random fields in at least three separate slides between 15 and 20 min after the addition of VEGF (30 ng/ml, PeproTech). Confocal images were obtained with a Zeiss Axiovert S100 TV and LSM 410 laser-scanning system. In some experiments, cells were fixed, permeabilized, and stained with 5 units of rhodamine phalloidin (Molecular Probes) prior to examination (12). Fluorescence ratio imaging was performed using Zeiss LSM software, version 3.98.

**Immunoprecipitation and Western Immunoblotting—**FLAG-p47**phox**-transfected HUVEC were rocked in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na3VO4, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na2VO3, 1 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) for 20 min, harvested, and sonicated once for 3 s. Insoluble material was removed by centrifugation at 4°C for 20 min at 10,000 × g prior to immunoprecipitation with anti-FLAG (M2, Sigma). Endogenous and adenovirally transduced p47**phox** were immunoprecipitated using mouse anti-p47**phox** (Upstate Biotechnology). Immunoblots were performed with antibodies against WAVE1 (Upstate Biotechnology), p47**phox** (Upstate Biotechnology) and a gift of Dr. Bernard Babior), PAK1 (Santa Cruz Biotechnology), Rac1 (Transduction Laboratories), and phosphotyrosine (Santa Cruz Biotechnology). p47**phox** Phosphorylation and Oxidant Production—HUVEC were transfected with pCINF-p47, recovered for 24 h, and then serum-starved for an additional 20 h. Culture medium was replaced with phosphate-free Dulbecco’s modified Eagle’s medium (Sigma) containing...
0.5 mCi/ml [32P]orthophosphate for 4 h. Cells were then stimulated with VEGF (30 ng/ml) for 15 min, and cell lysates were subjected to immunoprecipitation with anti-FLAG, PAGE separation, and autoradiography. A third of the immunoprecipitate was immunoblotted for p47<sub>phox</sub> to assess capture and loading. Oxidant production was assessed as oxidation of 2',7'-dichlorofluorescin diacetate (DCF, Molecular Probes). HUVEC were washed and loaded with DCF (10 μM) in Hanks’ balanced salt solution for 20 min at 37 °C. They were then washed and exposed to VEGF (30 ng/ml for 15 min), and DCF fluorescence was measured as described previously (12).

**RESULTS**

**WAVE1 Binds p47<sup>phox</sup>**—In its unstimulated state is thought to exist in a folded conformation with intramolecular interactions between its C terminus and its core SH3 domains (14). Phosphorylation of multiple serines within this C terminus results in the unmasking of these core binding domains. Therefore, we truncated the autoinhibitory C terminus and generated a WAVE1 fragment (100-442) that was cotransfected into HUVEC. Following stimulation with VEGF (30 ng/ml), ruffles were diminished in pCIN-p47-(153-286) or WAVE1-(100-442) expressing cells.

**Oxidants and WAVE1**

**FIG. 6.** Suppression of ruffles by p47-(153-286) and WAVE1-(100-442). HUVEC were cotransfected with actin-GFP and empty vector (a, b, e, and f), pCIN-p47-(153-286) (c and d), or pCIN-WAVE1-(100-442) (g and h). Following stimulation with VEGF (b, d, f, and h), ruffles were diminished in pCIN-p47-(153-286) or WAVE1-(100-442) expressing cells. i, number of ruffling cells was quantified using the actin-GFP transfectants. *p < 0.001 compared with control (first column). **p < 0.001 compared with VEGF + empty vector (second column).

**FIG. 7.** Coprecipitation of PAK1 and Rac1 with FLAG-p47. a, HUVEC were transfected with FLAG-p47 and stimulated with VEGF (30 ng/ml) for the indicated times, immunoprecipitated (IP) with anti-FLAG or irrelevant isotype control, and immunoblotted (IB) for PAK1 or Rac1 (upper panel). Blots were reprobed with anti-p47<sup>phox</sup> (second and fourth panels). Association of PAK1 and Rac1 with FLAG-p47 both appeared maximal within 15 min. b, FLAG-p47-transfected HUVEC were stimulated with VEGF for 15 min, immunoprecipitated with anti-FLAG, and then sequentially immunoblotted for WAVE1, PAK1, and then FLAG. Coprecipitation of WAVE1 and PAK1 with FLAG-p47 occurred following VEGF stimulation.

p47<sup>phox</sup> to assess capture and loading. Oxidant production was assessed as oxidation of 2',7'-dichlorofluorescin diacetate (DCF, Molecular Probes). HUVEC were washed and loaded with DCF (10 μM) in Hanks’ balanced salt solution for 20 min at 37 °C. They were then washed and exposed to VEGF (30 ng/ml for 15 min), and DCF fluorescence was measured as described previously (12).

**JNK and PAK Kinase Assays**—Following stimulation, cells were washed with ice-cold phosphate-buffered saline, scraped, pelleted, and lysed in lysis buffer. HA-JNK or PAK was immunoprecipitated with anti-HA or anti-PAK1 (Santa Cruz Biotechnology), and the immunocomplexes were washed twice with lysis buffer and once with kinase buffer. The immunocomplex kinase assay contained either GST-Jun (Santa Cruz Biotechnology) for JNK or myelin basic protein (Upstate Biotechnology) for PAK as a substrate in the presence of [γ-<sup>32</sup>P]ATP (PerkinElmer Life Sciences) at 30 °C for 30 min (13).
fused the remaining protein (p47-(1–298)) to Gal4-BD. Upon screening our HUVEC library, a clone representing the C-terminal 459 residues of WAVE1 in-frame with Gal4-AD was recovered. This clone survived auxotrophic and lacZ expression selection and was strongly positive upon retesting using a yeast-mating strategy. In addition, full-length WAVE1 bound robustly to GST-p47-(1–298) in vitro (Fig. 1a), indicating a direct interaction between the two proteins and suggesting the masking of the WAVE1 binding domain by the full-length p47phox protein. The two smaller labeled proteins in the WAVE1 translation reaction are presumably incompletely translated or partially proteolyzed WAVE1 truncations and were not present in reactions not containing the WAVE1 template (data not shown).

Using the yeast-mating technique, general binding domains were identified on both proteins. The WAVE1-binding region of p47phox was contained within the central tandem SH3 domains with the N-proximal domain (SH3a) in isolation binding more robustly than the isolated distal (SH3b) domain (Fig. 1c). Both p47-(1–205), containing the N-terminal Phox (PX) and SH3a domains, and p47-(205–390), containing the SH3b domain and...
C-terminal region, failed to bind WAVE1, presumably because of SH3 surface masking by proline-rich sequences within the adjacent PX and C-terminal regions, respectively. Interestingly, p47-(153–219,Arg-193), containing a Trp3Arg mutation disrupting the SH3a binding surface (12, 15) also bound WAVE1, suggesting a nonconventional mechanism of interaction perhaps unrelated to the SH3a surface.

p47phox in turn appeared to bind to the mid-region of WAVE1 (Fig. 1c). Again, the binding mechanism was not easily deduced. Neither residues 1–269 nor 270–599, which together span the entire protein, bound p47-(1–298). Similarly, neither 1–362 nor 363–559 bound p47-(1–298). An intact proline-rich region (residues 278–442) appeared to be necessary for binding as truncations not containing the full proline-rich region did not bind p47. GST pull down experiments also confirmed the ability of WAVE1-(278–442) and WAVE1-(100–442) to bind p47-(1–298) (Fig. 1d). Curiously, WAVE1-(270–559) bound p47-(1–298) in vitro but not in the yeast two-hybrid system, possibly due to interference with p47 binding by the N-terminal fused Gal4-AD in the WAVE1 truncation of the latter system.

VEGF Stimulates Interaction between WAVE1 and p47phox within Membrane Ruffles—As with its myeloid paralog WASP, WAVE1 functionally catalyzes Arp2/3-dependent actin nucleation and polymerization (16) and is thus found concentrated within membrane ruffles (17). In live HUVEC, we found that a DsRed-p47 fusion consistently colocalized with actin-GFP within membrane ruffles following VEGF stimulation, whereas DsRed-p47 was diffusely localized within the cytosol in unstimulated cells (Fig. 2). Specific localization to ruffles was demonstrated by fluorescence ratio imaging of cells cotransfected with DsRed-p47 and free EGFP (Fig. 3, a–c). In addition, the reversal of chromophores using p47-GFP and free DsRed also demonstrated relative concentration of p47phox in membrane ruffles (Fig. 3, d–f). Thus, localization of p47phox to ruffles did not appear to be a function of DsRed itself or of the increased cytosolic volume within ruffles. Interestingly, DsRed-p47 translocated to the nucleus in 15–30% of cells following VEGF stimulation (Fig. 2, d and f). The significance of this finding is unclear at this point but may reflect cotransport of p47phox with other mitogenic or survival factors to the nucleus.

In addition, endogenous WAVE1 coprecipitated with FLAG-p47 and recovery of WAVE1 was maximal at 15 min after stimulation with VEGF (Fig. 4a). This time course coincided with the onset of ruffling activity, which peaked at 10–15 min following VEGF addition, and subsided by 30 min. Furthermore, VEGF also increased coprecipitation of endogenous WAVE1 with endogenous p47phox (Fig. 4b). As expected, spatial
Figure 12. p47-(S303D,S304D,S328D) increases circular ruffles. HUVEC were infected with Ad-LacZ (a) or Ad-p47-(S303D,S304D,S328D) (Ad-p47-(SD)) (b and c) for 24 h prior to fixation and staining with rhodamine phalloidin. Numerous dorsal circular ruffles are seen in panel b. c. Z-plane reconstruction shows >10-μm height of circular ruffle. d. HUVEC were infected with Ad-p47-(SD) and immunoprecipitated with either irrelevant or anti-p47 antibodies and immunoblotted sequentially for WAVE1, PAK1, and p47phox. WAVE1 but not PAK1 specifically coprecipitated with p47-(SD). e. HUVEC were transfected with p47-GFP and stimulated with VEGF (30 ng/ml) for 10–20 min. Rare (<1%) cells displayed circular ruffles containing p47-GFP. Shown are four Z-plane images from apical (left) to basal (right) planes.
nase \textit{in vitro} (20), although its capacity to perform this function in intact cells has not been demonstrated. The formation of such a complex might therefore be expected to facilitate p47\textsubscript{phox} phosphorylation and consequent oxidant production. Indeed, we found that VEGF led to PAK1 activation within 5 min and caused phosphorylation of p47\textsubscript{phox} within 15 min, whereas kinase-dead dominant-negative (DN) PAK1-\(\text{R298A}\) blocked p47\textsubscript{phox} phosphorylation (Fig. 8, a–c). This ability of PAK1 to facilitate p47\textsubscript{phox} phosphorylation, either directly or indirectly, is consistent with recent studies of the NADPH oxidase suggesting that Rac1 activates unknown proximal signaling events leading to oxidase assembly independent from its direct involvement with the oxidase (21). In parallel, PAK1-\(\text{R298A}\) also blocked VEGF-induced oxidant production (Fig. 8d) and ruffle formation (Fig. 9). Furthermore, the membrane-permeant SOD mimetic MnTBAP also reduced VEGF-induced ruffling (Fig. 10), suggesting that the effects of endogenous p47\textsubscript{phox} occurred at least in part through oxidant production. Taken together, these data suggest a linear sequence of complex formation, p47\textsubscript{phox} phosphorylation, oxidant production, and membrane ruffling.

p47\textsubscript{-(S303D,S304D,S328D)} Increases Oxidant Production, Tyrosine Phosphorylation, and Ruffle Formation—To further implicate a specific role of p47-dependent oxidants in ruffle formation, we replaced three serines on p47\textsubscript{phox} known to be critically phosphorylated during activation of the phagocyte oxidase with aspartic acid residues to mimic phosphoserines. This triple mutant has been shown to cause constitutive activation of the NADPH oxidase in a cell-free reconstitution system (22). Adenoviral delivery of this mutant p47\textsubscript{phox} increased oxidant production of unstimulated HUVEC, whereas overexpression of the wild-type p47\textsubscript{phox} or the SH3a-defective p47\textsubscript{phox} did not have a significant effect on basal oxidant production (Fig. 11a). Overexpression of p47\textsubscript{-(S303D,S304D,S328D)} also increased total protein phosphotyrosine content, and this effect did not require the presence of VEGF and other growth factors (Fig. 11b). In contrast, overexpression of wild-type p47\textsubscript{phox} increased tyrosine phosphorylation only in the presence of growth factors, and p47\textsubscript{-(W193R)} overexpression had no appreciable effect with or without growth factors. Finally, overexpression of p47\textsubscript{-(S303D,S304D,S328D)} also resulted in the formation of circular dorsal ruffles in the absence of VEGF (Fig. 12, a–c), which were unusual in uninfected cells or cells infected with Ad-LacZ, Ad-p47, or Ad-p47\textsubscript{-(W193R)} (Fig. 12d and data not shown). WAVE1 also co precipitated with p47\textsubscript{-(S303D,S304D,S328D)} (Fig. 12d), consistent with activation-induced association of p47\textsubscript{phox} with WAVE1 and consequent ruffle formation. We could not demonstrate association of PAK1 with this complex (Fig. 12d), possibly because the active mutant of p47\textsubscript{phox} presumably enters the pathway distal to PAK1, thus bypassing it. The reason for the appearance of circular rather than peripheral linear ruffles is also not clear; however, PAK1 concentrates in dorsal circular ruffles and active PAK1 mutants such as H83L,H86L are known to induce dorsal ruffling, whereas active Rac1 mutants acting proximal to PAK1 more typically produce linear ruffles. Again, specific activation of the oxidase through an active p47\textsubscript{phox} mutant may bypass non-oxidase-dependent functions of Rac1 or elements upstream of Rac1, potentially biasing the response toward circular rather than linear ruffles. Notably, circular ruffles are also WAVE-dependent (23) and we noted that p47-GFP concent-
trated in the rare circular ruffles stimulated by VEGF (Fig. 12c), further suggesting that a common WAVE- and p47-dependent complex may be responsible for both linear and circular ruffles.

**VEGF Signaling Bifurcates Distal to PAK1 and Oxidant Production, Leading to JNK Activation and Ruffle Formation**—We confirmed that VEGF activated JNK (Fig. 13c), a MAPK known to mediate VEGF-dependent mitogenesis (24). Furthermore, PAK1-(K298A) effectively blocked VEGF-induced HA-JNK2 activation (Fig. 13c) as did the antioxidant MnTBAP (Fig. 13b). Thus, JNK activation similar to ruffle formation appears to lie downstream of PAK1 and oxidant production. To investigate whether JNK lies within a linear activation scheme leading to ruffle formation, we cotransfected DN-JNK2 with actin-GFP and found that DN-JNK2 did not diminish VEGF-induced ruffling (Fig. 14). Similar negative results were obtained with DN-JNK1 (data not shown). Therefore, VEGF signal pathways appear to diverge beyond oxidant production, leading independently to JNK activation and ruffle formation.

**DISCUSSION**

Cells commonly respond to extracellular signals with rapid architectural changes of the actin cytoskeleton tightly coupled with translocation of signals into the cell nucleus. This coordination between actin dynamics and signal transduction is exemplified by the angiogenic response of endothelial cells to VEGF, which initiates migration and proliferation of cells within a vascular sprout. The WASP/suppressor of cAMP receptor family proteins hold potential for integrating such cytoskeletal and signaling responses. The more intensely studied WASP and N-WASP have well known effects on actin nucleation and in addition bind signaling proteins such as Src and Tec family tyrosine kinases and adapters such as Pro-Ser-Thr phosphatase-interacting protein, Grb2, and Nck (25–28). Although WAVE1 has a clear role in the formation of Rac-induced ruffles and lamellipodia, its capacity to facilitate other signaling events is less clear. Most proteins shown to interact with WAVE1 appear to impart a negative regulatory function such as the Rac GTPase-activating-protein WRP and the inhibitory complex composed of p53-inducible mRNA-121, Nap125, and HSPC300 (29, 30). However, domains N-proximal to the actin and Arg23-binding C terminus are clearly necessary for the cytoskeletal function and targeting of WAVE1 (31, 32), suggesting recruitment of additional positive regulatory factors.

Our finding that VEGF stimulates association of WAVE1 with p47^phox^ suggests a possible linkage of WAVE1 function with oxidant-related cytoskeletal and signaling events. In support of this possibility, the reduction of oxidant levels through interventions upstream (DN-PAK1) or downstream (MnTBAP) of p47^phox^ or ectopic expression of the isolated binding domains of the two proteins was associated with a reduction in ruffle formation. In contrast, specific activation of p47^phox^ (p47-(S303D,S304D,S328D)) increased ruffle formation. The potential involvement of endogenous oxidants in actin dynamics is also suggested by recent studies demonstrating that NADPH oxidase inhibitors and O2 scavengers abolish actin monomer incorporation at the fast-growing filament barbed ends and block migration of endothelial cells (10). More specifically, oxidants derived from the NADPH oxidase have been linked to VEGF-induced endothelial cell migration and angiogenic behavior (33). The NADPH oxidase has also been implicated in MAPK activation by PDGF and tumor necrosis factor, both factors that induce membrane ruffling (2, 12). The association of WAVE1 with p47^phox^ may therefore reflect the existence of a molecular complex controlling both cytoskeletal and mitogenic events.

VEGF stimulates phosphorylation of endothelial p47^phox^, which is consistent with the unmasking of its core binding domains to allow binding to WAVE1. It is uncertain at this point, however, whether a single p47^phox^ molecule is able to bind simultaneously to WAVE1 as well as to the NADPH oxidase. Our data do not support a conventional SH3 surface-dependent interaction between p47^phox^ and WAVE1, allowing for the possibility that p22^phox^ and WAVE1 may simultaneously bind different surfaces within the core p47^phox^ region. Recent peptide walking data suggest that p22^phox^ itself may bind p47^phox^ through a non-SH3-dependent mechanism (34). Alternatively, the binding of p47^phox^ to WAVE1 and p22^phox^ may be sequential rather than simultaneous. In one scenario, p47^phox^ may first serve its function as an NADPH oxidase adapter and then leave the complex to recruit WAVE1. Indeed, once activated, the NADPH oxidase does not require p47^phox^ for O2 production (35). In an alternate scenario, p47^phox^ may first serve a novel role as a WAVE1 adapter by assisting membrane targeting of WAVE1 through its N-terminal PX domain, which binds to lipid products of phosphatidylinositol 3-kinase (36) before binding p22^phox^. Finally, dimerization of p47^phox^ within a WAVE1 complex would allow presentation of two SH3a surfaces per complex. That at least one of these possibilities operates in endothelial cells is strongly suggested by the colocalization of WAVE1, p47^phox^, and oxidant production within membrane ruffles.

Recruitment of p47^phox^ to a WAVE1 scaffold may reflect a general strategy of targeting oxidant production to critical subcellular sites as a means to gain signal specificity. In the context of VEGF signaling, the molecular targets of such oxidants are presently unknown. However, we found that expression of p47-(S303D,S304D,S328D) increased tyrosine phosphorylation of a number of proteins, consistent with the ability of O2 to reversibly inactivate protein tyrosine phosphatases (37). In this regard, the association of the tyrosine kinase c-Abl with the WAVE1 complex (16) may be expected to hold significance for both mitogenic and cytoskeletal signal pathways. Indeed, c-Abl appears to mediate both oncogenic Src and PDGF effects and PDGF fails to stimulate ruffles in c-Abl-null cells (38). Potential downstream c-Abl substrates include Crk and Enb, both linked to actin remodeling and survival signaling (39, 40).

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