Vascular Endothelial Growth Factor Receptor-3 Activity Is Modulated by Its Association with Caveolin-1 on Endothelial Membrane†

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ABSTRACT: Vascular endothelial growth factor receptor-3 (VEGFR-3) is constitutively expressed in lymphatic vessels and transiently in endothelial cells of blood vessels during angiogenesis. Here we report that VEGFR-3 localizes in the caveolae membrane of endothelial cells and co-immunoprecipitates with caveolin-1. Caveolin-1 silencing or its depletion from the cell membrane by cholesterol increases VEGFR-3 autophosphorylation, suggesting that caveolin acts as a negative regulator of VEGFR-3 activity. Receptor activation induces caveolin-1 phosphorylation on tyrosine residues including tyrosine 14. Cell treatment with Src or Abl inhibitors PP2 or STI571, prior to receptor stimulation, affects caveolin-1 phosphorylation without affecting receptor autophosphorylation, suggesting that both Src and Abl are involved in VEGFR-3-dependent caveolin-1 phosphorylation. Caveolin-1 phosphorylation in Src/Fyn/Yes knockout cells demonstrated that Abl phosphorylates caveolin-1 independently from Src family members. These results suggest a functional interaction between VEGFR-3 and caveolin-1 to modulate endothelial cell activation during angiogenesis.

Vascular endothelial growth factor receptor-3 (VEGFR-3) (also known as fms-like tyrosine kinase-4 or Flt-4) belongs to the family of endothelial-specific tyrosine kinase receptors that includes VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1). These receptors are activated by members of the vascular endothelial growth factor (VEGF)[1] family that includes, besides VEGF, the placental growth factor (PlGF) and VEGF-B, -C, and -D with partially overlapping receptor recognition (*1*, *2*).

VEGFR-3, which is activated by VEGF-C and VEGF-D, plays a central role in the differentiation of endothelial precursors and organization in primary vascular network (vasculogenesis) and the sprouting of new capillaries from preexisting vessels (angiogenesis) and homeostasis of lymphatic vessels (*1*−*3*). Vegfr-3 −/− embryos die of fluid accumulation in the pericardial cavity and cardiovascular failure at embryonic E9.5 with large vessels abnormally organized with defective lumens (*4*).

In adults, VEGFR-3 is constitutively expressed in lymphatic endothelial cells (*5*, *6*), in quiescent endothelial cells of fenestrated capillaries, and microvessels of several tissues (*7*−*9*). Endothelial cells transiently express VEGFR-3 at wound healing and in tumors (*10*−*13*). VEGFR-3 expression in endothelial cells of tumors might play a functional role, as its inactivation by monoclonal antibodies suppresses tumor growth by inhibiting the neoangiogenesis. Moreover, it has been hypothesized that during angiogenesis the transient expression of VEGFR-3 might negatively modulate VEGFR-2, contributing in this way to the maintenance of vascular integrity (*12*, *14*).

VEGFR-3 autophosphorylation recruits CRKI/II, GRB2, and SHC to different phosphorylated tyrosine residues within its C-teminal domain, which activate the second messengers JNK1/2, ERK1/2, PI3K, and AKT (*15*). VEGFR-3 can form heterodimers with and can be transphosphorylated by VEGFR-2 (*16*). However, the mechanism of VEGFR-3 activation remains elusive. Interestingly, it has been recently reported that VEGFR-2 is localized within caveolae and interacts with caveolin-1 in endothelial cells (*17*, *18*). Caveolae are discrete plasma membrane regions held together by caveolins. They consist in flask-shaped cell surface invaginations rich in cholesterol, sphingolipids, and sphingomyelin (*19*). Caveolae are present in several differentiated cell types, and endothelial cells express high levels of caveolin-1 (*20*), where it plays a fundamental role in the formation of caveolae (*21*). Caveolin-1 can be expressed in two alternative splicing variants, caveolin-1α and caveolin-1β, that lacks the first 31 amino acids (*22*). Although the function of caveolae remains elusive, several lines of evidence indicate an important role of caveolae and caveolin-1 in angiogenesis and endothelial cell functions. Caveolin-1 null mice suggest that it plays a major role in the cardiovascular function (*23*), endothelial albumin uptake, and transcytosis (*21*). Moreover, antisense oligonucleotide down-regulation of caveolin-1 impairs angiogenesis in vivo and in vitro (*24*). In proliferating endothelial cells VEGF treat-

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[1] Abbreviations: VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; CD, cyclodextrin; PNS, postnuclear supernatant; PM, plasma membrane; NCM, noncaveolae membrane; CM, caveolae membrane; HUVEC, human umbilical vein endothelial cells.

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ment leads to downregulation of caveolin-1 (*25*), but it is upregulated during endothelial cell differentiation, and its overexpression enhances capillary-like structure formation in Matrigel (*26*). Disruption of caveolin-1 leads to increased endothelial cell proliferation and sprouting (*27*, *28*).

Caveolae are putatively implicated in signal transduction (*29*, *30*). They have been shown to interact with several signaling proteins including cell receptors (EGFR, PDGF, VEGFR-2) and second messengers (Src, Fyn, heteromeric G proteins). In endothelial cells VEGFR-2 is localized in caveolae, and caveolin-1 overexpression inhibits VEGFR-2 signaling without altering VEGF-dependent VEGFR-2 activation (*18*).

Interaction of caveolin-1 with signal transduction molecules inhibits several signals including receptor-mediated activation of MAPK. On the other hand, cholesterol depleting agents demonstrated that several signaling proteins are caveaolae-dependent (*18*, *31*, *32*). Thus, caveolae might represent a way to aggregate signaling molecules into a plasma membrane microdomain, allowing a better molecular integration to switch the signaling above a certain threshold. The enrichment of signaling molecules into caveolae might be required for more precise on−off signaling pathways and/or to concentrate the signal transduction to specific cellular sites.

In this work we provide evidence that VEGFR-3 localizes in the caveolae of endothelial cells. VEGFR-3 and caveolin-1 co-immunoprecipitate, suggesting they form a complex. The functional interaction between VEGFR-3 and caveolin-1 was demonstrated by caveolin-1-dependent downmodulation of VEGFR-3 and by VEGFR-3-dependent tyrosine phosphorylation of caveolin-1. Moreover, we found that c-Abl and c-Src can independently phosphorylate caveolin-1 in a VEGFR-3-dependent manner. These results suggest that caveolin-1 is involved in the regulation of VEGFR-3 function in endothelial cells.

EXPERIMENTAL PROCEDURES

Immunohistochemistry. Animals used in this study were treated and sacrificed with procedures approved by the ethical committee and according to the European community guidelines. For histological analysis of adult skin, 6-week-old C57BL6/J mice were sacrificed, and samples of skin were dissected, fixed in 3% paraformaldehyde, dehydrated, and embedded in paraffin. Sections (8 μm) were immunostained by using rabbit polyclonal antibodies against VEGFR-3 and caveolin-1 (sc 321 and sc-894 from Santa Cruz Biotechnology, Santa Cruz, CA). Peroxidase activity was developed with 3-amino-9-ethylcarbazole (Sigma), and the sections were counterstained with hematoxylin. The tyramide signal amplification (Perkin-Elmer Life Sciences) was used to enhance staining. Images were captured using a Nikon DMX1200 digital camera from a Nikon Eclipse E600 microscope.

To generate tumors, Balb/C nude mice were injected in the mammary fat pads with MDA-MB-435 human breast cancer cells (2×10^6) in culture medium. Animals were killed after 4−5 weeks, and the tumors were removed and embedded in OCT (Tissue-Tek). Cryostat sections (10 μm) were cut, fixed in 3% paraformaldehyde, permeabilized with 0.5% Triton X-100 in PBS, and blocked with 1% bovine serum albumin (BSA) in PBS. Specimens were stained using rabbit anti-caveolin-1 polyclonal antibodies and rat anti-VEGFR-3 monoclonal antibody AFL4 (eBioscience, San Diego, CA). Primary antibodies were revealed with Alexa Fluor-568 and Alexa Fluor-488 secondary antibodies (Molecular Probes). Specimens were mounted in Mowiol 4-88 (Calbiochem, San Diego, CA), and fluorescent images were captured using a Leica TCS SP2 laser scanning confocal microscope.

Transfections and Infections. All of the Abl constructs (pSGT-Abl-wt, the constitutively active pSGTAbl-PP, and the kinase dead pSGT-Abl-Kin$^-$) (*33*, *34*) were kindly provided by D. Barila' (University of Rome "Tor Vergata"). The c-Src expression vectors were purchased from Upstate Biotechnology: pUSE-Src (K296R/Y528F) (dominant negative, DN) and pUSE-Src (Y529F) (dominant positive, DP).

Caveolin-1 α and β cDNA were amplified from HUVEC mRNA using the BcaBEST RNA PCR kit (Takara Bio Inc.) and the following oligonucleotides: forward α-caveolin-1, 5′-GAGAAAGCTTGCCGCCGCCATGTCTGGGGGCAAATACGTAG-3′, forward β-caveolin-1, 5′-GAGAAAAGCTTGCCGCCGCCATGGCAGACGAGCTGAGCGAG-3′, and reverse, 5′-GAGACTCGAGTATTTCTTTCTGCAAGTTGATGCGG-3′). The amplified cDNAs were subsequently cloned with *Hin*dIII−*Xho*I in pcDNA3/Neo (Invitrogen) in frame with the FLAG epitope at the 3′ end. All constructs were confirmed by sequencing.

The VEGFR-3 expression vector was obtained by subcloning *Hin*dIII−*Xba*I with the full-length cDNA of human VEGFR-3 [pECE-FLT4, kindly provided by Dr. D. Birnbaum (Institut National de la Santé et de la Recherche Médicale, INSERM, Marseille, France)] in pcDNA3.1/Hygro(+) (Invitrogen).

The transient transfections were performed in 293 cells using Polyfectamine reagent (Qiagen). PAE stable clones were obtained by transfecting cells with pcDNA3-Neo-caveolin-1 and VEGFR-3 constructs and selecting with G418 and hygromycin (GIBCO).

shRNA expressing lentiviral vectors (Open Biosystems) contains the 21 bp caveolin-1 sequence, 5′-CGACGTGGTCAAGATTGACTT-3′, and the green fluorescence protein (GFP) sequence, 5′-GCCACAAGTTCAGCGTGTCT-3′. The chimera receptor E-R3, the kinase dead mutant E-R3 Y1068F, the retroviral and lentiviral vectors, and virus preparation were described in ref *15*.

Cell Cultures. HUVEC were grown on gelatin-coated Petri dishes in M199 medium (GIBCO) supplemented with 20% FBS, 50 units/mL penicillin−streptomycin, 10 units/mL heparin, and 100 μg/mL brain extract. PAE cells were grown in HAMS F12 medium (GIBCO) supplemented with 10% FBS, 50 units/mL penicillin−streptomycin, and 2 mM L-glutamine. Drug selections of PAE cells were performed at 0.4 mg/mL G418 and 0.2 mg/mL hygromycin (GIBCO). 293 (ATCC CRL-1573) and SYF (ATCC CRL-2459) were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% FBS, 50 units/mL penicillin−streptomycin, and 2 mM L-glutamine. Where indicated cells were starved in the corresponding medium, 0.25% BSA, 0.5% FBS, for 16 h and induced with 1−10 ng/mL EGF (Sigma, St. Louis, MO) or 200 ng/mL VEGF-D.

When indicated HUVEC cells were treated for 60 min with 1 μM STI571 in DMSO (Novartis, Basel, Switzerland), 1

μM PP2 in DMSO (Calbiochem, San Diego, CA), diluted in DMSO, or for 45 min with 10 mM β-cyclodextrin (CD) before induction with EGF or VEGF-D.

Protein Extracts, Immunoblotting, and Immunoprecipitation. Caveolae membranes were purified by the detergent-free method (*35*). Following transfections or stimulations, cells were lysed in RIPA plus buffer [50 mM Tris-HCl (pH 7.2), 100 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 50 mM NaF, 2 mM sodium orthovanadate, 1 mM dithiothreitol (DTT), 60 mM octyl glucoside (Sigma) antiprotease cocktail; Roche], and cell debris was eliminated by centrifugation at 21000*g* at 4 °C for 10 min. For immunoblotting 50 μg of protein was separated by sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis (PAGE), blotted onto nitrocellulose membrane, and detected with specific antibodies. The following antibodies were used for immunoblotting: anti-VEGFR3 (sc 321), VEGFR-2 (sc-504), P-tyrosine (sc-7020), caveolin-1 (sc-894), P-caveolin-1 (Tyr14) (sc-14037), c-Abl (sc-131), c-Src (sc-18) purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-ERK1/2 and P-ERK1/2 (Cell Signaling Technology, Beverly, MA), and anti-CRK clone 22 (Transduction Laboratories, BD Biosciences, San Jose, CA).

For immunoprecipitations, protein extracts were incubated for 1 h at 4 °C with the following antibodies coupled to Dynabeads Protein A or Dynabeads Pan Mouse IgG (Dynal Biotech, Oslo, Norway): anti-caveolin-1 (sc-894; Santa Cruz Biotechnology, Santa Cruz, CA), anti-VEGFR3 (sc 321; Santa Cruz Biotechnology, Santa Cruz, CA), anti-CRK clone 22 (BD Biosciences, San Jose, CA), anti-Abl antibody Ab-3 (Calbiochem, San Diego, CA), and anti-FLAG (Sigma). Immunoprecipitations shown in Figure 2B were performed by extracting proteins with lysis buffer [25 mM Tris-HCl (pH 7.2), 0.5% NP40, 0.25% sodium deoxycholate, 1 mM NaF, 1 mM sodium orthovanadate, 60 mM octyl glucoside (Sigma), 1 mM EDTA, 1 mM EGTA, antiprotease cocktail (Roche)] and using the Exactacrutz F (sc-45043) immunoprecipitation kit following the manufacturer's instructions.

Abl Kinase Activity Assay. c-Abl proteins were immunoprecipitated from total cell extracts with anti-Abl antibody Ab-3 (Calbiochem, San Diego, CA) coupled to Dynabeads Pan Mouse IgG. Beads were washed two times with RIPA buffer, two times with RIPA reduced buffer [50 mM Tris-HCl (pH 7.2), 100 mM NaCl, 1% Triton X-100, 1 mM NaF, 2 mM sodium orthovanadate, and 1 mM dithiothreitol (DTT)], and two times with kinase assay buffer [25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 10 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, and 0.3% BSA]. A 20 μL volume of kinase assay mix [1 μg of glutathione *S*-transferase GST-CRK-SH3 (aa 121−226), 0.2 μL of [γ-^{32}P]ATP (Amersham), 10 mM ATP in kinase assay buffer] was added, and the mixture was incubated at 25 °C for 20 min. The kinase reaction was stopped by adding SDS sample buffer and analyzed by SDS−PAGE.

RESULTS

VEGFR-3 and Caveolin-1 Are Expressed in Lymphatic and Blood Endothelial Cells. To study a functional interaction between caveolin-1 and VEGFR-3, we first analyzed in vivo whether these two molecules were expressed in the same cells. Endothelial cells of blood vessels were previously shown to express high levels of caveolin-1 (*20*). To investigate in vivo the expression of caveolin-1, we stained adjacent sections of mouse skin with anti-VEGFR-3 (Figure 1A) and anti-caveolin-1 (Figure 1B) antibodies. We observed caveolin-1 staining both in blood and in lymphatic endothelial cells while VEGFR-3 staining was revealed only in lymphatic vessels in accordance with previous reports (*5*, *6*). As VEGFR-3 is upregulated in endothelial cells undergoing angiogenesis (*10*−*13*), we also analyzed VEGFR-3 expression in solid tumors obtained by injecting MDA-MB-435 breast cancer cells into the mammary pads of nude mice. After 4−5 weeks, when tumors formed new capillary vessels mice were sacrificed and analyzed. As shown in Figure 1C,D, endothelial cells of newly forming tumor vessels were positive for both caveolin-1 and VEGFR-3 staining, demonstrating that VEGFR-3 and caveolin-1 are coexpressed.

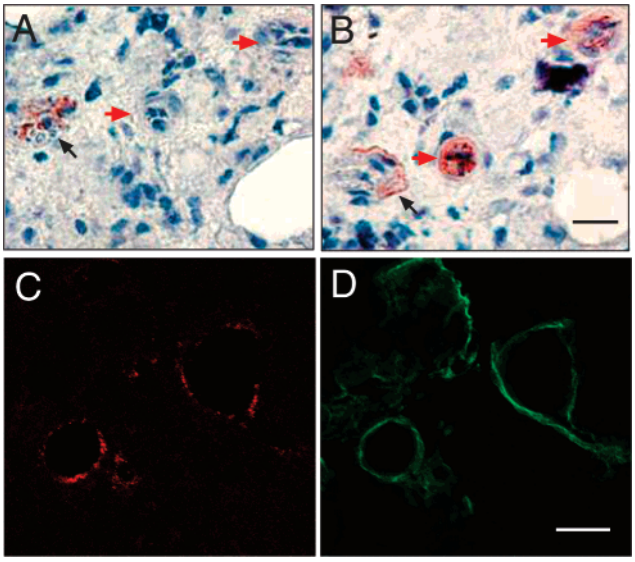

FIGURE 1: VEGFR-3 and caveolin-1 colocalize in normal skin lymphatic vessels and in tumoral angiogenesis. (A) Sample of adult murine skin stained with anti-VEGFR-3 polyclonal antibodies. (B) Adjacent section stained with anti-caveolin-1 polyclonal antibodies. The black arrow indicates a lymphatic vessel, which stains for VEGFR-3 and caveolin-1. Blood vessels (red arrows) stain only for caveolin-1. Scale bar = 40 μm. (C) Cryostat section of newly forming vessels of a tumor grown in nude mice. Tissue was stained with anti-VEGFR-3 monoclonal antibodies. (D) The same section as in panel C stained with anti-caveolin-1 polyclonal antibodies. VEGFR-3 and caveolin-1 colocalize in newly forming tumor vessels. Scale bar = 20 μm.

VEGFR-3 Is Localized into Caveolae and Functionally Interacts with Caveolin-1. To verify whether VEGFR-3 and caveolin-1 colocalize on the endothelial cell membrane, caveolae were purified from primary human umbilical vein endothelial cells (HUVEC) by using a detergent-free method (*35*). As shown in Figure 2A, the different fractions containing either the postnuclear supernatant (PNS), the plasma membrane (PM), the noncaveolae membrane (NCM), or the caveolae membrane (CM) were separated by electrophoresis and immunoblotted. Caveolin-1 was largely enriched in the CM fraction, which was also enriched for VEGFR-3. Western blot of VEGFR-2 was performed as a positive control (*18*). Formation of a VEGFR-3 caveolin-1 complex was confirmed by co-immunoprecipitation experiments. HUVEC extracts were immunoprecipitated using rabbit polyclonal antibodies against the C-terminus of VEGFR-3. The immunoprecipitated was revealed for VEGFR-3 and for

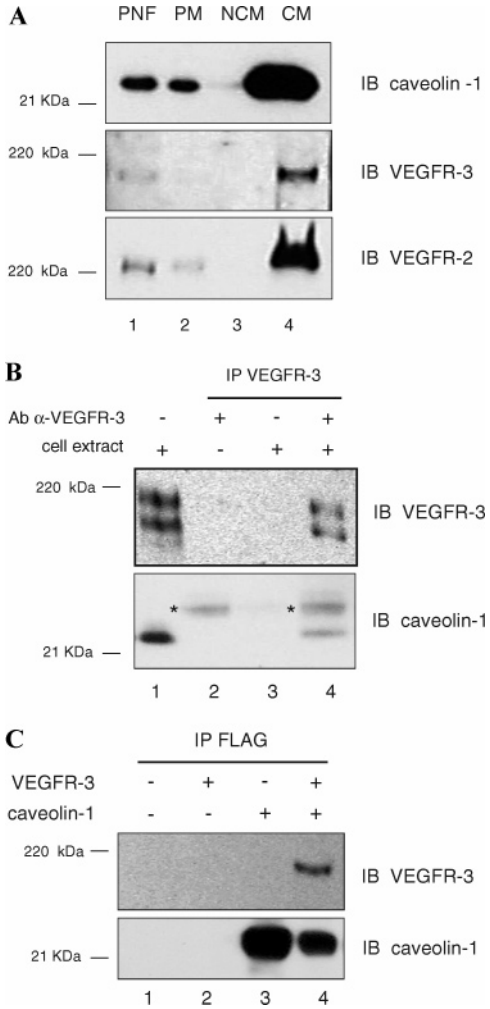

FIGURE 2: VEGFR-3 receptors are concentrated in caveolae and interact with caveolin-1. (A) Caveolae were isolated from subconfluent, primary human umbilical vein endothelial cells (HUVEC) on Optiprep gradients (*35*), and 5 μg of protein from either the postnuclear supernatant (PNS), the plasma membrane (PM), the noncaveolae membrane (NCM), or the caveolae membrane (CM) fractions was separated by SDS−PAGE and immunoblotted (IB) with the indicated antibodies. The caveolae membrane fraction was enriched for caveolin-1, VEGFR-3, and VEGFR-2. (B) VEGFR-3 and caveolin-1 coimmunoprecipitate. HUVEC extracts were immunoprecipitated with anti-VEGFR-3 antibodies and revealed with anti-VEGFR-3 or anti-caveolin-1 as indicated. Input in 1/10 of the IP. (C) 293 cells were transfected with expression vectors for VEGFR-3 and/or FLAG-tagged caveolin-1. After 48 h cells were collected and lysed. Protein samples were immunoprecipitated (IP) with anti-FLAG monoclonal antibody. Proteins were eluted from beads with SDS sample buffer, separated by SDS−PAGE, and blotted with the indicated antibody.

caveolin-1 (Figure 2B). To further confirm the interaction between VEGFR-3 and caveolin-1, 293 cells were transiently transfected with vectors expressing full-length wild-type VEGFR-3 together with a C-terminal FLAG-tagged caveolin-1. The whole cell lysates were immunoprecipitated with an antibody against the FLAG and immunoblotted for VEGFR-3 or caveolin-1 (Figure 2C).

We further analyzed the functional interplay between VEGFR-3 and caveolin-1, measuring whether caveolin-1 affected VEGFR-3 autophosphorylation. Stable PAE cell clones expressing VEGFR-3 were transfected either with a mock vector, caveolin-1α, or caveolin-1β (Figure 3A) and treated with 200 ng/mL recombinant VEGF-D. The receptor

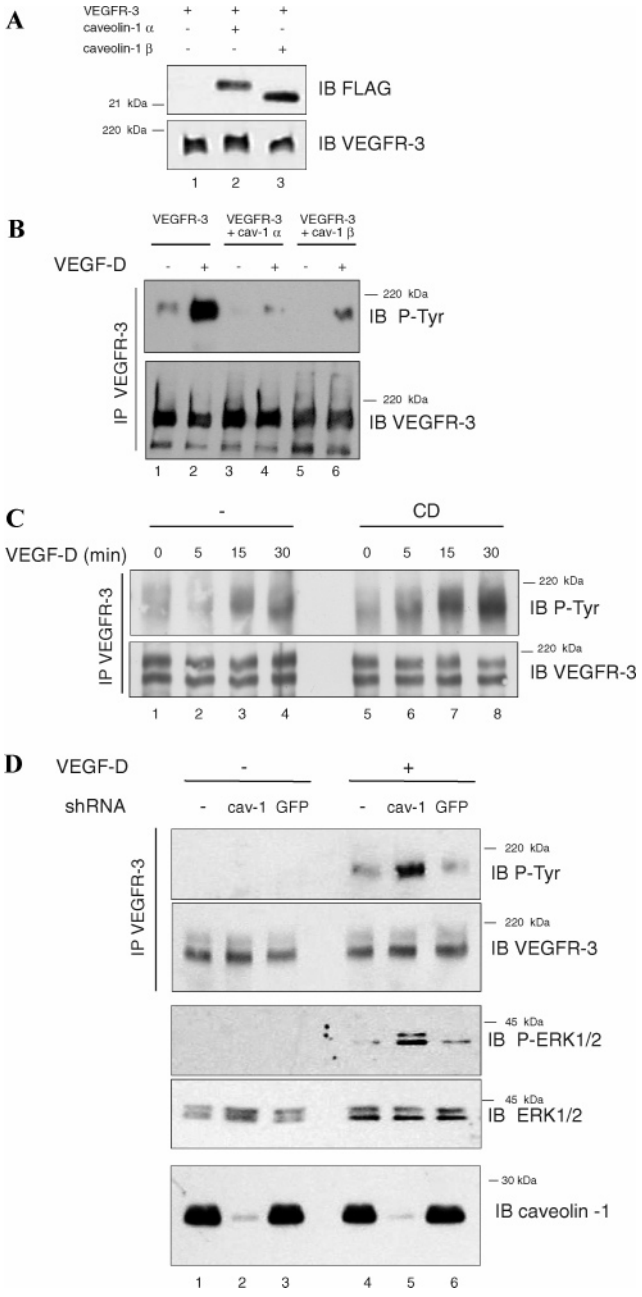

FIGURE 3: Caveolin-1 regulated VEGFR-3 autophosphorylation. (A) Expression levels of ectopic VEGFR-3 and caveolin-1 isoforms in porcine aortic endothelial cell (PAE) stable clones stably transfected with human VEGFR-3 alone, VEGFR-3 and caveolin-1α, or VEGFR-3 and caveolin-1β as indicated. (B) Overexpression of either caveolin-1 isoform downregulates VEGFR-3 autophosphorylation. Protein extracts from cells treated with 200 ng/mL VEFG-D for 15 min were subjected to immunoprecipitation with polyclonal anti-VEGFR-3 and then blotted with the indicated antibodies. (C) Caveolae depletion activates VEGFR-3 autophosphorylation. Serum-starved HUVEC were incubated for 45 min with 10 mM β-cyclodextrin (CD) and then treated with 200 ng/mL VEGF-D for the times indicated. (D) VEGFR-3 activation by VEGF-D in mock-infected cells and in cells expressing caveolin-1 shRNA. Serum-starved cells were treated with 200 ng/mL VEFG-D for 15 min and lysed. Protein extracts were subjected to immunoprecipitation and blotted with the indicated antibodies. The lower panel shows a Western blot analysis of caveolin-1 in mock and silenced cells.

autophosphorylation was then analyzed by immunoprecipitation of VEGFR-3 with C-terminal specific antibodies and immunoblotted with anti-phosphotyrosine antibody. The

ectopic expression of either caveolin-1α or caveolin-1β repressed VEGF-D-dependent VEGFR-3 autophosphorylation (Figure 3B). In addition, in HUVEC caveolin-1 removal by cholesterol depletion obtained with cyclodextrin (CD) (*31*) showed an enhanced VEGFR-3 authophosphorylation (Figure 3C). As the results observed CD could be nonspecific, we also analyzed VEGF-R3 phosphorylation in cells silenced for caveolin-1. VEGFR-3 autophosphorylation as well as ERK1/2 phosphorylation was strongly enhanced in cells infected with a viral vector expressing caveolin-1 shRNA (Figure 3D), demonstrating that caveolin-1 modulates VEGFR-3 activity.

VEGFR-3 Stimulates Phosphorylation of Caveolin-1. Next we determined whether the close association between VEGFR-3 and caveolin-1 induced VEGFR-3-dependent phosphorylation of caveolin-1. HUVEC were transduced with the wild-type EGFR-VEGFR-3 (E-R3) chimeric receptor, with the E-R3-Y1068F kinase inactive mutant, or with the mock vector. This chimera was used to analyze the VEGFR-3 downstream signaling avoiding the interference of VEGFR-2 as VEGF-D induces both VEGFR-2 and VEGFR-3 (*36*). Cells were treated with 10 ng/mL EGF at different time points. Caveolin-1 was immunoprecipitated from cell lysates and analyzed with an anti-phosphotyrosine antibody as well as with polyclonal antibodies recognizing the phosphorylation of tyrosine 14 as this tyrosine residue appears to be the most characterized site of caveolin-1 phosphorylation (*37*, *38*). Following E-R3 activation there was a strong increase of tyrosine phosphorylation of caveolin-1 with a peak at 15 min (Figure 4A). Caveolin-1 phosphorylation alters its mobility with an apparent molecular mass between 20 and 30 kDa as previously described (*39*). The phosphorylated bands were not observed in mock-infected cells or in HUVEC transduced with the kinase inactive receptor, thus suggesting that these caveolin-1 phophorylations are E-R3-dependent.

VEGFR-3-Dependent Tyrosine Phosphorylation of Caveolin-1 Is Mediated by c-Src and c-Abl. c-Src has been previously shown to phosphorylate caveolin-1 in vivo and in vitro (*37*, *39*−*41*). The tyrosine 14 of caveolin-1 is a well-described site of Src phosphorylation. This amino acid lies within a consensus for Abl phosphorylation (Y-X-X-P), and it has been shown to be indeed a substrate for v-Abl and c-Abl following oxidative stress (*42*, *43*). Analysis with kinase inhibitors revealed that the Src specific inhibitor PP2 (*44*) reduced, although did not eliminate, E-R3-dependent caveolin-1 phosphorylation (Figure 4B). A similar result was obtained with the Abl-specific inhibitor STI571 (also known as Gleevec) (*45*), suggesting that both Src and Abl are involved in caveolin-1 phosphorylation. Importantly, this effect was not due to nonspecific effects of the inhibitors since they did not alter E-R3 autophosphorylation or ERK1/2 activation (Figure 4C). The involvement of Abl in the VEGFR-3-dependent phosphorylation of caveolin-1 was unexpected as VEGFR-2-dependent caveolin-1 phosphorylation was previously shown to be only dependent on Src (*18*). Therefore, we further investigated Abl involvement in VEGFR-3-dependent caveolin-1 phosphorylation. Time course analysis revealed that Abl was phosphorylated already at 2 min after EGF treatment in HUVEC transduced with E-R3, while it was not induced in mock-transfected cells (Figure 5A). As CRK is a known Abl target, we analyzed Abl activation by an in vitro kinase assay with cell extracts

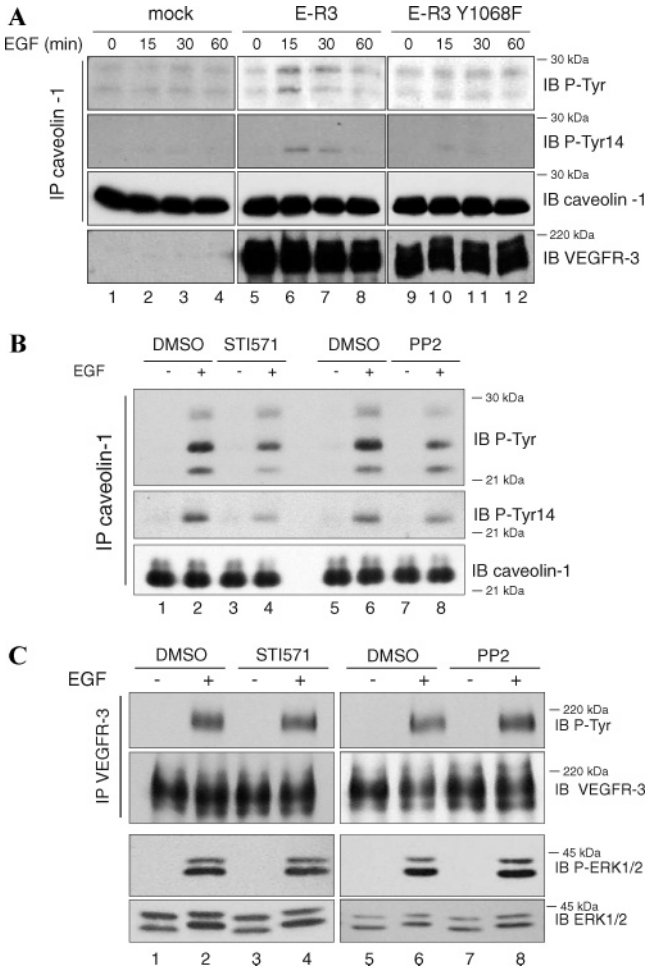

FIGURE 4: VEGFR-3 stimulates Src- and Abl-dependent phosphorylation of caveolin-1. (A) HUVEC cells were transduced with mock vector or with receptor chimera E-R3 wild-type (E-R3) or kinase dead (E-R3 Y1068F) and treated with 10 ng/mL EGF for the times indicated. (B, C) Abl and Src inhibitors specifically affect caveolin-1 phosphorylation. HUVEC cells transduced with E-R3 were preincubated for 1 h with the Abl-specific inhibitor STI571 (1 μM in DMSO), the Src-specific inhibitor PP2 (1 μM in DMSO), or DMSO and treated with 10 ng/mL EGF for 15 min.

precipitated for Abl using as substrate GST-CRK-SH3. This assay demonstrated that Abl is activated in cells expressing the wild-type receptor but not in cells transduced with mock vector (Figure 5B). Moreover, Abl-dependent CRK phosphorylation was inhibited by STI571 in cells, thus confirming that Abl is induced in a E-R3-dependent manner (Figure 5C).

To demonstrate that Abl phosphorylates caveolin-1 independently from Src, we analyzed caveolin-1 phosphorylation in mouse fibroblasts derived from a triple knockout for Src, Yes, and Fyn (SYF) (*46*). These cells were transduced with E-R3 chimera and then treated with EGF. SYF cells could still be induced by E-R3-dependent caveolin-1 phopshorylation albeit to a lower level (Figure 6A), and the expression of constitutively active Abl induces caveolin-1 phosphorylation (Figure 6B). These experiments demonstrated that E-R3-dependent caveolin-1 phosphorylation can be obtained in the absence of members of the Src family. This result was further confirmed by 293 cell transfection with constructs expressing kinase dead or constitutively active Abl or Src which showed a strong caveolin-1 phosphorylation with either kinase (Figure 6C) demonstrating that both Src and Abl phosphorylate caveolin to comparable levels. Taken

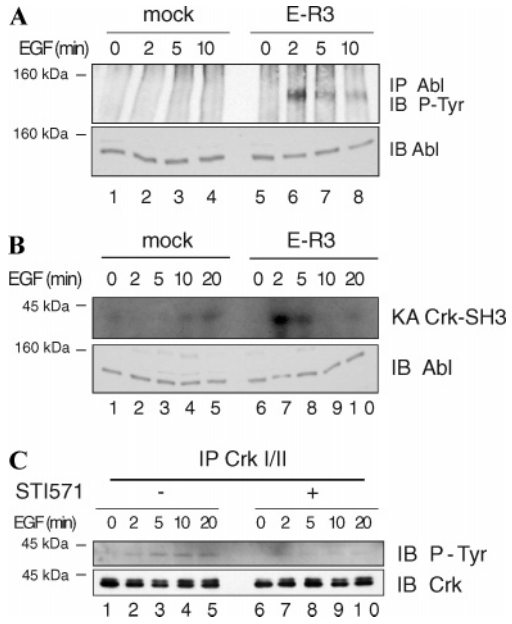

FIGURE 5: VEGFR-3 induces c-Abl activation. (A) HUVEC cells transduced with mock vector or E-R3 were treated with 10 ng/mL EGF for the times indicated. Cells were lysed in RIPA buffer, immunoprecipitated with anti-Abl antibody, and blotted with anti-P-Tyr. Abl phosphorylation was induced already at 2 min after EGF treatment. (B) The Abl activity was measured by in vitro kinase assay (KA) after c-Abl immunoprecipitation with the GST-CRK-SH3 (aa 121−226) protein as a substrate in the presence of [γ-^{32}P]-ATP. (C) Endogenous CRK is phosphorylated by Abl. The phosphorylation of endogenous CRK II was measured by CRK immunoprecipitation and subsequent immunoblot with anti-P-Tyr- and anti-CRK-specific antibodies. The Abl activity was completely inhibited by preincubating cells with 1 μM STI571 (lanes 6−10).

together, these experiments allow us to conclude that Abl contributes to VEGFR-3-dependent caveolin-1 phosphorylation independently from Src.

DISCUSSION

VEGFR-3 signaling plays an important role in angiogenesis and lymphangiogenesis. However, the mechanism of its activation in endothelial cells is still incompletely understood. Several reports demonstrated the involvement of caveolae in the regulation of tyrosine kinase receptors including the highly related VEGFR-2. In this study we report that VEGFR-3, together with VEGFR-2, is highly enriched in caveolae membrane domains of endothelial cells, suggesting that VEGFR-3 requires this compartmentalization for its proper function. Our histochemical analysis demonstrated the presence of caveolin-1 not only in vascular but also in lymphatic endothelial cells. Importantly, we could observe the colocalization of caveolin-1 with VEGFR-3 in normal lymphatic vessels as well as in vascular endothelial cells undergoing active angiogenesis as in these cells VEGFR-3 is induced during angiogenesis. In the caveolae VEGFR-3 forms a complex with caveolin-1 as both proteins can be co-immunoprecipitated. The formation of the VEGFR-3 and caveolin-1 complex in endothelial cells must play a functional role on VEGFR-3 signaling as caveolin-1 overexpression determined a reduction of VEGFR-3 autophosphorylation while its depletion by RNAi or CD induced the opposite effect. The inhibitory activity of caveolin-1 on the receptor autophosphorylation and signaling might reflect a direct interaction between VEGFR-3 and caveolin-1, which could be

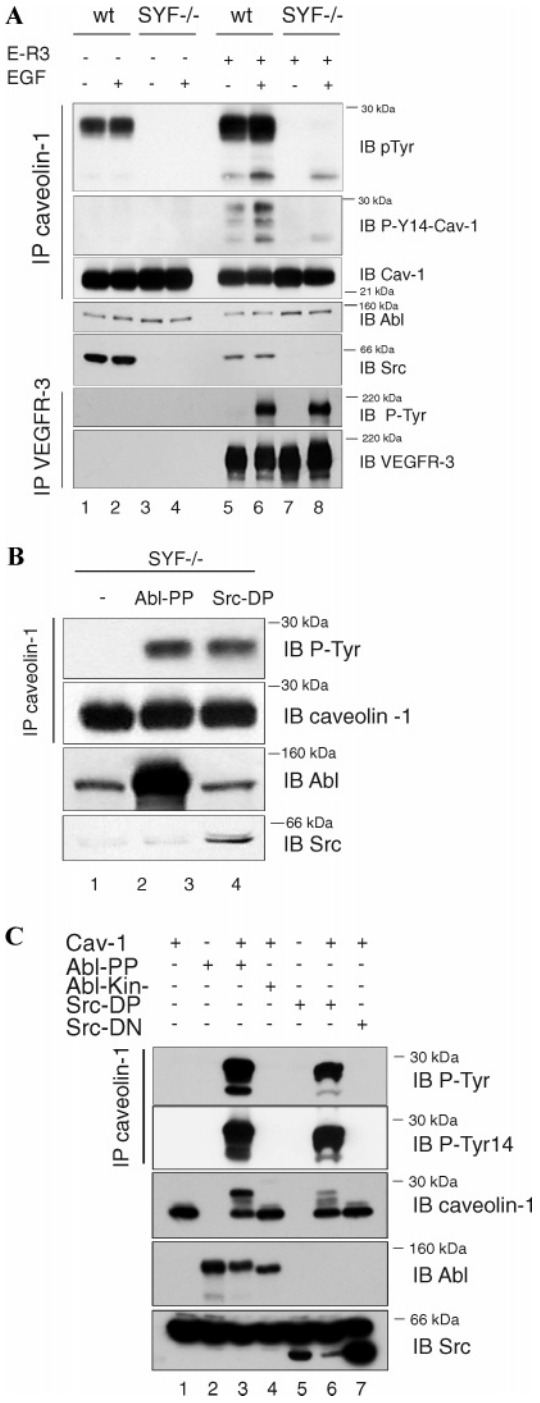

FIGURE 6: VEGFR-3 induces Abl-dependent caveolin-1 phosphorylation in the absence of Src. (A) E-R3 induces caveolin phosphorylation independently of Src family members Src, Yes, and Fyn. Src, Yes, and Fyn triple knockout cells (SYF−/−) and control wild-type cells (wt) were transduced with mock vector (lanes 1−4) or E-R3 (lanes 5−10) chimera and induced for 15 min with 10 ng/mL EGF. (B) SYF−/− cells were transfected with the constitutively active Abl-PP and Src-DP. Both kinases induced a robust caveolin-1 phosphorylation. Proteins immunoprecipitated with anti-caveolin-1 or anti-VEGFR-3 and total cell extract were immunoblotted with the indicated antibodies. Constitutively active Abl and Src induce comparable levels of caveolin phosphorylation. (C) 293 cells were transfected with expression vectors for caveolin-1α and the constitutively active (Abl-PP and Src-DP) or kinase dead (Abl-Kin− and Src-DN) kinases as indicated. Phosphorylation of caveolin-1 induced a strong retardation in SDS−PAGE migration.

mediated by the putative caveolin-1 binding domain that has been described for EGFR and other tyrosine kinase receptors

(*47*). In fact, a caveolin-binding motif (ΦXΦXXXXΦXXΦ, where Φ is an aromatic amino acid) is present within the kinase domains of both VEGFR-3 and VEGFR-2 with identical amino acid sequence (WSFGVLLWEIF). VEGFR-3 inhibition by caveolin-1 might play a regulatory function by limiting the receptor activity. The localization of the receptor within the caveolae might provide a control of the receptor activation modulating its efficiency and the specificity of the downstream signaling. In this respect it is interesting to note that caveolin-1 itself is a VEGFR-3 substrate, which was phosphorylated on tyrosine residues. VEGFR-3-dependent caveolin-1 phosphorylation could have different functional roles. In fact, it has been previously demonstrated that tyrosine 14 can recruit proteins containing SH2 or PTB domains like Grb-7 (*48*), C-terminal Src kinase (Csk) (*38*). In addition, caveolin-1 phosphorylation might be involved in the structural function of caveolae. Thus, following the receptor activation caveolin-1 phosphorylation might be required for the reorganization of the caveolae to permit the formation of the activated complex and/or to downmodulate the signaling after receptor activation disengaging the activated complex containing the receptor and inducing the internalization of the receptor with a molecular mechanism similar to the caveolae internalization after cell detachment from the extracellular matrix (*49*).

The mechanism of VEGFR-3-dependent caveolin-1 phosphorylation needs further studies. Our experiments demonstrate that caveolin-1 phosphorylation is mediated by Src or Abl activated independently by VEGFR-3. In fact, specific inhibitors of either of these kinases reduced only partially caveolin-1 phosphorylation. Importantly, we observed for the first time, in a VEGFR family member, the Abl-specific activation with fast kinetics as well as Abl-dependent phosphorylation of caveolin-1 upon VEGFR-3 activation. As Src-dependent phosphorylation of caveolin-1 was previously demonstrated for VEGFR-2 and other tyrosine kinase receptors, these results provide evidence that Abl plays a role in caveolin-1 activation independently from the presence of Src. Although our experiments do not permit distinguishing whether these two kinases induce a different pattern of caveolin-1 phophorylation, it is likely that the different distribution beween Src and Abl might play a role in caveolin-1 phosphorylation function.

In conclusion, the identification of a functional interaction between caveolin-1 and VEGFR-3, which is transiently expressed when endothelial cells undergo active angiogenesis, strongly suggests that the interplay between caveolin-1 and VEGFR-3 plays a relevant role during the activation of endothelial cells that takes place in the angiogenic processes.

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