Ligand-induced vascular endothelial growth factor receptor-3 (VEGFR-3) heterodimerization with VEGFR-2 in primary lymphatic endothelial cells regulates tyrosine phosphorylation sites

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Summary

Vascular endothelial growth factors (VEGFs) regulate the development and growth of the blood and lymphatic vascular systems. Of the three VEGF receptors (VEGFR), VEGFR-1 and -2 are expressed in blood vessels. VEGFR-3 is expressed mainly on lymphatic vessels but it is also upregulated in tumor angiogenesis. Although VEGFR-3 is essential for proper lymphatic development, its signal transduction mechanisms are still incompletely understood. Transphosphorylation of activated, dimerized receptor tyrosine kinases is known to be critical for the regulation of kinase activity and for receptor interaction with signal transduction molecules. In this study, we have identified five tyrosyl phosphorylation sites in the VEGFR-3 C-terminal tail. These sites were used both in VEGFR-3 overexpressed in 293 cells and when the endogenous VEGFR-3 was activated in lymphatic endothelial cells (LECs). Interestingly, VEGF-C stimulation of LECs induced also the formation of VEGFR-3/VEGFR-2 heterodimers, in which VEGFR-3 was phosphorylated only at three of the five sites while the two most C-terminal tyrosine residues appeared not to be accessible for the VEGFR-2 kinase. Our data suggests that the C-terminal tail of VEGFR-3 provides important regulatory tyrosine phosphorylation sites with potential signal transduction capacity and that these sites are differentially used in ligand-induced homo- and heterdimeric receptor complexes.
Introduction

The receptor tyrosine kinase vascular endothelial growth factor receptor 3 (VEGFR-3, previously denoted fms-like tyrosine kinase-4; Flt-4) is essential for the development of the blood and lymphatic vasculature. Inactivation of the \textit{VEGFR-3} gene in mouse embryos leads to a disturbed vascular development resulting in an irregular vessel pattern and a reduced cross-sectional area of large vessels. The embryos die at embryonic day 9.5 due to fluid accumulation in the pericardial cavity and cardiovascular failure (3).

In adult tissues, VEGFR-3 is expressed primarily on lymphatic endothelial cells (34) and appears to exert its major functions within this system. Thus, inactivating missense point mutations in one \textit{VEGFR3} allele lead to chronic lymphedema (13). Further, overexpression of a soluble VEGFR-3 in mice leads to regression of lymph vessels and features characteristic of lymphedema, without any apparent effects on the blood vasculature (18). In several tumor models, overexpression of the VEGFR-3 ligand VEGF-C increases lymphangiogenesis and promotes spread of metastases (15, 22, 29). The same effect is achieved by overexpression of VEGF-D, another VEGFR-3 ligand (30).

VEGFR-3 is to some extent expressed also on quiescent vascular endothelial cells, primarily in fenestrated capillaries (17, 27, 34). Very low levels can be occasionally detected in the blood vascular endothelium of wound granulation tissue, and in vessels stimulated with VEGFs (5, 35). Further, the endothelium of angiogenic blood vessels of several tumors express VEGFR-3 (26, 31, 35). These results suggest that VEGFR-3 could be involved in aspects of angiogenesis in adults.

The VEGFR-3 is similar in the overall structure to the VEGFR-1 and VEGFR-2 (24); the extracellular, ligand-binding domain is composed of seven immunoglobulin (Ig)-like folds and the intracellular domain is characterized by an interrupted tyrosine kinase domain. In contrast to the other VEGF receptors, the VEGFR-3 extracellular domain is cleaved within the 5th Ig-homology domain; the resulting two polypeptides are held together by a disulfide bridge (25). In humans, but not in mice, a retroviral insertion between the last two exons of VEGFR-3 (9) results in two splice variants of the VEGFR-3 of which the shorter form lacks 65 amino acids in the cytoplasmic tail (7, 23).

Activation of the VEGFR-3 tyrosine kinase appears to follow the consensus scheme for receptor
tyrosine kinases. Ligand binding results in receptor dimerization and sequential activation of the intrinsic kinase activity. Trans-phosphorylation between the partners in the dimer regulates kinase activity and creates docking sites for signaling molecules with characteristic domains such as Src homology (SH)-2 or phosphotyrosine binding (PTB) domains. The specificity of binding is determined by the sequence adjacent to the phosphotyrosine residue. The activated VEGFR-3 associates with adaptor proteins Shc and Grb2 via tyrosine 1337 (6, 25). Moreover, VEGFR-3 activation leads to protein kinase C (PKC)-dependent activation of Erk-1 and -2, implicated in cell proliferation. Furthermore, VEGFR-3 mediates the activation of protein kinase B/Akt, implicated in cell survival (19). In accordance, VEGFR-3 transduces signals resulting in proliferation, migration and survival of lymphatic endothelial cells (19). In this report, we have examined the potential phosphorylation of tyrosine residues in the long form of the activated VEGFR-3 and provide evidence for phosphorylation in five positions in the cytoplasmic tail. In addition, we show complex formation between VEGFR-3 and the related VEGFR-2 in primary lymphatic endothelial cells. In the heterodimeric configuration, VEGFR-2 failed to phosphorylate VEGFR-3 on two of the carboxy-terminal sites, Y1337 and Y1663. This has implications for the VEGFR-3 signal transduction properties.

Experimental procedures

Growth factors and antibodies

The growth factors used were epidermal growth factor (EGF, #100-15, Peprotech, Rockyhill, NJ), human VEGF (#100-20, Peprotech, Rockyhill, NJ) human VEGF-C (Thr103-Leu215; (11)) ligand for VEGFR-2 and VEGFR-3. The antibodies used were mouse anti-VEGFR-3 (clones 9D9F9, 2E11D11; (12, 19)); rabbit anti-VEGFR-2 (RS-2; (33)), anti phosphotyrosine 4G10 #05-321, Upstate, NY, rabbit anti-V5 #A190-120A, Bethyl Laboratories, Mongomery TX, and anti-actin, # sc-1615, Santa Cruz CA. Rabbit anti-human podoplanin antibodies were kindly provided by Donscho Kerjaschki, Vienna, Austria.
Cell lines and cell culture

Porcine aorta endothelial (PAE) cells, stably overexpressing VEGFR-3 or VEGFR-2 were maintained in F12/10% fetal calf serum (FCS). Human 293T cells were used for transient expression of VEGFR-3 and were maintained in DMEM/10% FCS. Primary lymphatic endothelial cells (LECs) were separated from human dermal microvascular endothelial cells as previously described (19) using antibodies against podoplanin. The cells were cultured on gelatin-coated plastic in endothelial basal medium (EBM, CC-3121, Clonetics, Walkersville, Maryland) supplemented with 5% FCS, 30 µg/ml endothelial growth culture supplement (ECGS, E-7060, Sigma, St Louis, Missouri), 10 ng/ml EGF and 10 ng/ml VEGF-C (Thr103-Ile225).

Generation of mutated VEGFR-3

VEGFR-3 tyrosine mutants were generated by the GeneEditor in vitro Site-Directed Mutagenesis kit (Promega) using oligonucleotides in which one nucleotide change in the tyrosine encoding sequence was introduced, resulting in Tyr-> Phe amino acid change in the protein sequence.

VEGFR-3 kinase dead (R3-KD; R1041P and R978G) mutants were generated as above using oligonucleotides which introduced desired nucleotide changes in the VEGFR-3 kinase-domain encoding sequence. The R1041P represents a mutation found in lympedema (14) while in the K879G mutant the ATP-binding Lys has been changed into Gly. Both mutant proteins were found to be kinase-inactive when expressed in 293T-cells (14).

Transient transfections

Vectors (pcDNA3.1/Zeo, Invitrogen AB, Lidingö, Sweden) encoding wild type and mutant VEGFR-3 were transfected into human 293T cells using the calcium phosphate method. In brief, 6 x 10^6 cells in 10 cm Petri dishes were treated with 25 µM chloroquine for 1.5 h. Vector cDNA (4-10 µg) in 250 mM CaCl_2, was mixed with 2x concentrated Hank’s balanced salt solution (HBSS), incubated for 20 min and then added to the cells. Five to six h later, cells were treated for two min with 10% glycerol in medium. The cells were harvested 48 h post transfection.
**Immunocomplex kinase assay and SDS-PAGE**

The cells were starved over night in serum-free medium supplemented with 0.1% bovine serum albumin, and treated for 8 min with or without VEGF or VEGF-C using 50 ng/ml, washed in Tris-buffered saline (TBS)/100 µM Na$_3$VO$_4$. on ice. The cells were lysed in ice-cold NP40 lysis buffer (1% Nonidet P40, 20 mM Heps, pH 7.5, 150 mM NaCl, 10% glycerol, 2.5 mM EDTA, 10 Ie/u aprotinin/ml, 1 mM PMSF, and 100 µm Na$_3$VO$_4$). An aliquot of the cell lysate was saved for control blotting.

Lysates were clarified by centrifugation and incubated for 2 h on ice with in-house anti-VEGFR-3 antibodies. The mouse and rabbit antibodies were precipitated with protein G-sepharose (#17-0618-01, Amersham Biosciences, Uppsala, Sweden) and protein A-sepharose (Immunosorb A, Medicago AB, Uppsala, Sweden) respectively. The precipitate was washed three times in lysis buffer and twice in kinase buffer (20 mM Heps, 10 mM MgCl$_2$, 2 mM MnCl$_2$, 0.05% Triton-X-100). The precipitate was incubated for 10 min in kinase buffer containing 20 µCi [ýt$^{32}$P]ATP (Amersham Biosciences, Uppsala, Sweden) at 37°C and heated in sample buffer (8% SDS, 0.4 M Tris-HCl pH 8.0, 1 M sucrose, 10 mM EDTA, 0.02% bromphenol blue, 4% β-mercaptooethanol). The samples were separated by SDS-PAGE, using 7% polyacrylamide gel, transferred to a nitrocellulose membrane and detected by a BioImager (BioImager, BAS-1800II Fujifilm, Tokyo, Japan) screen that was subsequently scanned using the BioImager.

**Immunoblotting**

Samples were prepared essentially as described above but without the kinase reaction, separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membranes were incubated with indicated primary antibodies and subsequently, with HRP-conjugated secondary antibodies. Immunoreactive sites were visualized using an enhanced chemiluminescence detection system (Amersham Biosciences, Uppsala, Sweden).
Phosphopeptide mapping

The detailed procedure has been described previously (1, 32). Briefly, the phosphorylated bands on the membrane from the in vitro complex assay were localized using the BioImager scan, cut out and treated for 30 min at 37°C in 0.5% polyvinylpyrrolidone in 100 mM acetic acid, washed four times in H$_2$O, digested by 1 µg trypsin (#V542A, Promega, Madison, Wisconsin) in 200 µl 50 mM NH$_4$CO$_3$ at 37°C over night. The supernatant was lyophilized in a centrifugal vacuum concentrator and dissolved in 50 µl performic acid (formic acid: 30% H$_2$O$_2$; 9:1) and incubated for 1 h at room temperature, diluted with 500 µl H$_2$O and frozen to -135°C. The frozen samples were lyophilized again and dissolved in 50 mM NH$_4$CO$_3$ supplemented with 1 µg trypsin and incubated over night at 37°C. One hundred and forty µl pH 1.9 buffer (2.2% formic acid and 7.8% acetic acid in H$_2$O) was added and any particles were precipitated by centrifugation. One hundred and eighty µl of the supernatant was lyophilized and dissolved in 7 µl pH 1.9 buffer. The samples were centrifuged and 6 µl of each sample was gently dried onto a cellulose covered thin-layer chromatography glass plate (#1.05716, Merck, Darmstadt, Germany) as a spot of about 5 mm in diameter, localized 5 cm from the left side and 3 cm from the bottom of the plate. The peptides were separated by electrophoresis (x-axis) using pH 1.9 buffer and the plate was dried. Separation in the second dimension (y-axis) was performed by ascending chromatography using isobutyric acid buffer (62.5% isobutyric acid, 1.9% n-butanol, 4.8% pyridine, 2.9% acetic acid in H$_2$O) over night. The plate was dried and the peptides with incorporated $^{32}$P were detected by the BioImager. Using the resulting phosphopeptide map for localization, cellulose containing peptide spots of interest were scraped off the chromatography plate. The peptides were extracted by pH 1.9 buffer, lyophilized, and used for Edman degradation or phosphoaminoacid analysis.

Edman degradation and radio amino acid sequencing

For Edman degradation, phosphopeptides were coupled to sequelon-AA membranes (Millipore, Sundbyberg, Sweden) and sequenced using a Gas Phase sequencer (Applied Biosystems, Foster City, California). The fractions were spotted onto thin-layer chromatography plates, detected by BioImager.
and analysed by the BioImager software.

**Phospho-amino acid analysis**

The samples extracted from the thin-layer plates were hydrolyzed in 6 M HCl at 110°C and lyophilized, dissolved in H₂O and lyophilized again. The samples were dissolved in 7 µl pH 1.9 buffer supplemented with non-radioactive phosphorylated serine, threonine and tyrosine as markers and spotted onto a thin-layer chromatography plate. Electrophoresis using first the pH 1.9 buffer (x-axis) and subsequently the pH 3.5 buffer (acetic acid 5% and pyridine 0.5% in H₂O; y-axis) provided two-dimensional separation of the hydrolyzed amino acid residues. The dried plates were sprayed with ninhydrin for detection of the marker phospho-amino acid residues. The plate was dried and the incorporated ³²P was visualized using the BioImager. By overlaying the resulting image with the ninhydrin pattern of the marker amino acids, the identity of the amino acid residues with incorporated ³²P was determined.

**Results**

**Phosphorylation of VEGFR-3.**

The intracellular domain of the long form of VEGFR-3 contains 16 tyrosine residues. Of these, six tyrosine residues are located in the carboxy-terminal tail (denoted Y1230, Y1231, Y1265, Y1333, Y1337 and Y1363; Fig.1). Trypsin digestion of the VEGFR-3 intracellular domain results in the generation of up to 17 tyrosine-containing peptides, of which four are derived from the carboxy-terminal tail (Table 1). We wished to determine which of the carboxy-terminal tyrosine residues that could serve as potential phosphorylation sites. Stimulation of PAE cells expressing VEGFR-3 with VEGF-C, resulted in strong induction of VEGFR-3 phosphorylation, as estimated in an in vitro immunocomplex kinase assay (Fig. 2A). Trypsin-digested peptides of the receptor were separated by electrophoresis according to the charge/mass ratio (x-axis) and by liquid chromatography according to hydrophobicity (y-axis), creating a phosphopeptide map. In this analysis, a corresponding increase in phosphorylation was detected in an analysis of trypsin-generated peptides from the ligand-stimulated VEGFR-3 (Fig. 2B).
**Position of phosphorylated residues**

Peptides giving reproducible spots in the phosphopeptide maps were extracted, hydrolyzed in hydrochloric acid and separated by two-dimensional electrophoresis on thin-layer plates. Unlabeled phosphoamino acid residues served as references (indicated by circles in the insets in Fig. 2C). Peptides displaying tyrosine phosphorylation (encircled by in Fig. 2B) were subjected to Edman degradation. Chemical identification of amino acid residues was not feasible due to the minute amounts of protein in the assay. Instead, the material in each cycle was spotted individually on thin-layer chromatography plates and the $^{32}$P-content in each fraction was quantified after exposure and detection using a BioImager. The positions of the radioactive peaks were the basis for tentative identification of a tryptic peptide containing a tyrosine residue in the corresponding position. Peptides from some distinct spots displayed identical radiosequences (spots denoted c in Fig. 2). This may be due to serine phosphorylation of the corresponding peptide, which contains five serine residues. Serine phosphorylation was indeed detected in the leftmost spot (data not shown). The predicted change in the phosphopeptide map position due to such a modification is in compliance with the observed positions. An alterative explanation to multiple spots is partial oxidation of cysteine residues with consequences for the charge and thereby the migration position of the peptide.

**Analyses of VEGFR-3 tyrosine to phenylalanine mutants**

The above results indicated that all tyrosine residues in the carboxy-terminal tail, with the possible exception of Y1333, were phosphorylated. To further exploit these findings, we created six receptors variants, point mutated in the carboxy-terminal tail, each with a single amino acid exchange from tyrosine to phenylalanine. These receptors were denoted Y1230F-R3, Y1231F-R3, Y1265F-R3, Y1333F-R3, Y1337F-R3 and Y1363F-R3. Phosphopeptide mapping of the mutant receptors transiently expressed in 293 cells, confirmed the preliminary identification of the phosphopeptides (Fig. 3). In the phosphopeptide map of Y1265F-R3 one spot was lost whereas the map of Y1337F-R3 showed loss of three spots (Figure 3). The corresponding spots in the Y1333F-R3 map were shifted...
in the y-direction indicating increased hydrophobicity, in agreement with the expected change as a result of the exchange of tyrosine for phenylalanine. The Y1337 residue was still phosphorylated in this peptide. The peptides containing Y1230/Y1231 and Y1363 were not fully separated, but analysis of this region in the phosphopeptide map (boxed in Fig. 3A) by scanning densitometry confirmed that loss of tyrosine phosphorylation as a consequence of the different mutations resulted in the expected phosphopeptide pattern (Fig. 3H).

**VEGFR-2 and VEGFR-3 heterodimerization in primary cells.**

We wished to verify that the phosphorylation pattern of VEGFR-3 overexpressed in PAE or 293 cells mimicked that of the endogenously expressed VEGFR-3 in primary lymphatic endothelial cells (LECs). Fig. 4 shows that the phosphopeptide map of LEC-derived VEGFR-3 was essentially indistinguishable from that of the overexpressed recombinant receptor, confirming the relevance of our approach. Minor differences in migration positions of a collection of spots in the right margin were anticipated, as there had been some variations in this region of the TLC plate also between repeated maps of VEGFR-3 overexpressed in the 293 cells.

The immunocomplex kinase assay of VEGFR-3 immunoprecipitated from VEGF-C-stimulated LECs demonstrated that a phosphorylated protein of 220 kDa was co-immunoprecipitated by the VEGFR-3-specific antibodies. We hypothesized that this component could correspond to VEGFR-2. This was confirmed by phosphopeptide mapping (data not shown). To investigate under which conditions the association between VEGFR-2 and VEGFR-3 occurred, LECs were treated with VEGF, specific for VEGFR-2, or VEGF-C, a ligand for both receptors. Immunoprecipitation with antibodies specific for the respective receptors, followed by immunocomplex kinase assay, demonstrated that the association was evident only after stimulation with VEGF-C (Fig. 5A). The specificity of the antibodies was confirmed by immunoprecipitation and blotting of cell lysates derived from PAE cells overexpressing either VEGFR-2 or VEGFR-3 (Fig. 5B). The different levels of kinase activity and the different properties of the antisera used precluded a determination of the relative stoichiometry of heterodimers versus homodimers in the VEGF-C-treated LECs.
Distinct pattern of phosphorylation of VEGFR-3 in the heterodimeric configuration

To examine if VEGFR-3 was phosphorylated similarly in the homodimeric and the heterodimeric configuration, a kinase-dead mutant VEGFR-3 in which the ATP-binding lysine is mutated (R1041P; here denoted R3-KD) was expressed alone or in combination with VEGFR-2 or VEGFR-3 in 293 cells. The V5-tagged R3-KD was specifically recognized by anti-V5 antibodies, ensuring that the analysis was focused on R3-KD dimerized with kinase active VEGFR-2 or VEGFR-3. In this set-up, phosphorylation of R3-KD was dependent on the co-expressed kinase active receptors. As shown in Fig. 6, wt-VEGFR-3-mediated phosphorylation of R3-KD resulted in a phosphopeptide map very similar to the previous VEGFR-3 maps (see Fig. 1). In contrast, the map of R3-KD phosphorylated by VEGFR-2 lacked spots corresponding to peptides containing tyrosine residues Y1337 and Y1363. Repeating the analysis using another kinase inactive VEGFR-3 (K879G; (14)) gave identical results (data not shown).

Discussion

Tyrosine phosphorylation sites in receptor tyrosine kinases regulate both kinase activity and interaction with signal transduction molecules. Thus, identification of these sites is of fundamental importance in understanding the signaling of a specific receptor. We show that five (Y1230, Y1231, Y1265, Y1337 and Y1363) of the six tyrosine residues in the C-terminal tail of VEGFR-3 are potential phosphorylation sites. The role of most of these sites in signal transduction downstream of VEGFR-3 remains to be determined. Y1337 is required for association of the Shc/Grb2 complex to VEGFR-3 (6) and this interaction has been linked to the transforming capacity of the receptor overexpressed in fibroblasts. In accordance, the short form of the VEGFR-3 that lacks the tyrosines Y1333, Y1337 and Y1363, is unable to mediate fibroblast transformation.

Our preliminary results suggest that the tyrosine residues 1063 and 1068 in the second kinase domain become phosphorylated upon receptor activation (data not shown). The positions of these
sites corresponds to those previously implicated in positive regulation of tyrosine kinase activity, e.g. Y1054 and Y1059 in VEGFR-2 (20). We therefore suggest that Y1063 and Y1068 in VEGFR-3 serve a positive regulatory role in the activation of the VEGFR-3 kinase. Of the remaining tyrosine residues in the intracellular domain of VEGFR-3, five are located in the first and second parts of the kinase domain, and three are located in the juxtamembrane domain (Y812, Y830 and Y833). Tyrosine residues at positions conserved relative to Y812 are found both in VEGFR-1 (Y794) and VEGFR-2 (Y801) (10). All three VEGFR-3 juxtamembrane tyrosine residues are contained within the same tryptic peptide. Edman degradation of this peptide has not allowed an unambiguous conclusion on the phosphorylation of these tyrosine residues.

The kinase insert domain plays an important role in signal transduction by the PDGF receptors. This sequence is of varying length in different the receptor tyrosine kinases and it is usually not conserved between otherwise related receptors, such as the PDGF α- and β-receptors (2), which has prompted the suggestion that the kinase insert is important in receptor type-specific signaling. It is therefore interesting that neither the VEGFR-1 nor the VEGFR-3 kinase insert contain any tyrosine residues. In contrast, the VEGFR-2 kinase insert contains three tyrosine residues of which at least one is a phosphorylation site (20).

Our analysis of VEGFR-3 tyrosine phosphorylation in primary lymphatic endothelial cells allowed the following conclusions: 1) The phosphorylation pattern of VEGFR-3 was faithfully reproduced between the receptor overexpressing cells and primary LECs; 2) In these primary cells, VEGF-C, but not VEGF, treatment induced formation of VEGFR-2 and VEGFR-3 heterodimers; 3) VEGFR-3 phosphorylation site usage was altered in the heterodimeric configuration. Thus, the two most C-terminal tyrosine residues in VEGFR-3 are substrates only for the VEGFR-3. VEGFR-3 in adult blood vessels and at least some lymphatic endothelia occurs in areas with VEGFR-2 expression (28, 34), indicating that VEGFR-2/VEGFR-3 heterodimers may form in vivo. Interestingly, recent data suggest that VEGFR-3 modulates the sensitivity to VEGFR-2 signaling to promote vascular integrity in blood vascular endothelial cells co-expressing the two receptors (16, 21). It is possible that such a cross-talk between the receptors is dependent on heterodimerization.
Growth factors of the VEGF and PDGF families are dimeric proteins in which each monomer contributes one receptor binding site. The different PDGF variants induce homo- or heterodimers of the PDGF receptors in a manner dictated by the receptor-specificity of the monomers. Similarly, treatment with VEGF, which is a common ligand for VEGFR-1 and VEGFR-2, induces receptor heterodimerization and functional signaling units (8). PDGF α- and β-receptor heterodimers have been shown to have signal transduction properties distinct from the respective α-α and β-β homodimeric forms, due to receptor tyrosine phosphorylation specific for the heterodimeric receptors (4). The differences in the phosphorylation site pattern between homo- and heterodimeric VEGFR-3 suggest that the signal transduction properties and biological function are distinct for the heterodimerized VEGFR-3. In particular, Shc and Grb-2, which are known to bind to Y1337 (25), are most likely not substrates for heterodimeric VEGFR-3. VEGF-C is produced as a 60 kDa protein with affinity for VEGFR-3 but poor affinity for VEGFR-2 (11). A stepwise proteolysis of VEGF-C results in a 20 kDa fragment with high affinity for both receptors are derived. Thus, VEGF-C proteolysis provides a mechanism whereby VEGFR-2/VEGFR-3 heterodimerization and, in turn, VEGFR-3 signalling, could be modulated.

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References


Figure legends

Figure 1. Schematic representation of VEGFR-3. Positions of tyrosine residues are indicated by the red dots in the intracellular domain and by numbers in the carboxy-terminal tail.

Figure 2. Phospho-peptide mapping, radioactive amino acid sequencing and phosphoamino acid analyses of VEGFR-3. PAE cells overexpressing VEGFR-3 were treated with or without VEGF-C for 8 min. A. Immunoprecipitated VEGFR-3 was $^{32}$P-labeled by immunocomplex kinase assay, subjected to SDS-PAGE and transferred to nitrocellulose membrane. B. Trypsin treatment of the VEGFR-3 band on the membrane was followed by separation in two dimensions on a cellulose thin-layer plate. Encircled peptides (a-d) were present in the VEGF-C treated cells only. One of the peptides migrated in three positions (three spots labeled c). C. Radioactive amino acid sequences of peptides a-d in the B panel. Numbers of consecutive cycles in the Edman degradation (1) and tentative alignment of tyrosine residues in tryptic VEGFR-3 peptides with the radioactive peaks (2) are indicated. The corresponding phosphoamino acid analyses are inserted in each panel a-d. Positions of reference phospho-serine (S), phospho-threonine (T) and phospho-tyrosine (Y) are encircled.

Figure 3. Phospho-peptide maps of wild type and mutated VEGFR-3. Phospho-VEGFR-3 immunoprecipitates were subjected to phosphopeptide mapping. A. The wild type phosphopeptide map. B-G. Phosphopeptide maps of the point mutated receptors. Peptides lost in the mutant maps as compared to the wild type map are indicated by arrows. Peptides with shifted positions as a consequence of the mutation are indicated by arrowheads. H. Phospho-peptides that appeared to be lost in Y1230F-R3, Y1231F-R3 and Y1363F-R3 were not fully separated from the other peptides in the region boxed in panel A. Densitometric scanning of the corresponding regions in the phosphopeptide maps of these receptors was carried out using the BioImager software. The wild type receptor and Y1265F-R3 were used as references for the densitometric curves. Arrows indicate major changes for each mutant VEGFR-3.
Figure 4. Analysis of VEGFR-3 phosphorylation in primary LECs. A. LECs with or without VEGF-C treatment were subjected to in vitro complex kinase assay. The expected three VEGFR-3 bands were detected, as indicated (Fig. 2A). The arrow indicates a band of 220 kDa, which was identified as VEGFR-2. B. VEGFR-3 bands from the VEGF-C-stimulated LECs were cut out and subjected to phosphopeptide mapping. The left (green) and the right (red) panels indicate phosphopeptide maps created from VEGFR-3 transiently expressed in 293T cells and primary LECs, respectively. These two phosphopeptide maps are overlaid in the middle panel.

Figure 5. VEGFR-2 co-precipitates with VEGFR-3 after stimulation with VEGF-C. A. Primary LECs were treated with VEGF (binds to VEGFR-1 and VEGFR-2) or VEGF-C (binds to VEGFR-2 and VEGFR-3). The receptors were immunoprecipitated with receptor-specific antibodies and subjected to immunocomplex kinase assay (upper panel). The migration rates of VEGFR-2 and the three bands corresponding to VEGFR-3 are indicated. Lower panel: Control blot of cell lysate showing equal amounts of actin in the different samples. B. Demonstration of the specificity of the antibodies used for immunoprecipitation in A, using PAE cells overexpressing VEGFR-2 or VEGFR-3. 1) indicates the mixture of the two anti-VEGFR-3 antibodies 9D9F9, 2E11D11, and 2) indicates the use of the 9D9F9 antibody alone.

Figure 6. VEGFR-2 heterodimerized with VEGFR-3 fails to phosphorylate VEGFR-3 on Tyr1337 and Tyr1363. V5 epitope-tagged kinase-dead VEGFR-3 (R3-KD) was expressed alone or in combination with either wt VEGFR-3 or wt VEGFR-2 in 293T cells. Cells stimulated with VEGF-C were lysed and R3-KD was immunoprecipitated and used for phosphopeptide mapping. Spots representing phosphopeptides containing tyrosine residues that failed to become phosphorylated by VEGFR-2 are encircled by the dashed lines.
**Table 1**

Predicted tyrosine-containing peptides

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<td>8</td>
<td>TFEEFPMTPTTY1265K</td>
<td>12</td>
<td>Carboxy-terminal tail</td>
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<tr>
<td>9</td>
<td>GGQVFY1333NSEY1337GELSEPSEEDHCSPSAR</td>
<td>6, 10</td>
<td>Carboxy-terminal tail</td>
</tr>
<tr>
<td>10</td>
<td>VTFFTDNSY1363</td>
<td>9</td>
<td>Carboxy-terminal tail</td>
</tr>
</tbody>
</table>

1) In tryptic peptide
2) The first 15 amino acids from kinase insert.
3) Possible alternative peptides of a single amino acid stretch.
Juxtamembrane Kinase domain 1
Kinase insert
Kinase domain 2
Carboxyterminal tail

Short form splice site

Fig. 1
Fig. 2

A

B

Control
VEGF-C

<table>
<thead>
<tr>
<th>VEGFR-3</th>
<th>VEGFR-3</th>
<th>VEGFR-3</th>
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<tbody>
<tr>
<td>IP: anti VEGFR-3</td>
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</table>

VEGFR-3

Phosphorylated amino acid

32P activity [arbitrary unit]

Number of consecutive cycles of Edman degradation

1) Tentative position of VEGFR-3 tyrosine residues

1) Number of consecutive cycles of Edman degradation

2) Tentative position of VEGFR-3 tyrosine residues
Ligand-induced vascular endothelial growth factor receptor-3 (VEGFR-3) heterodimerization with VEGFR-2 in primary lymphatic endothelial cells regulates tyrosine phosphorylation sites
Johan Dixelius, Taija Mäkinen, Maria Wirzenius, Marika Karkkainen, Christer Wernstedt, Kari Alitalo and Lena Claesson-Welsh

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