Functional interaction of VEGF-C and VEGF-D with neuropilin receptors

Terhi Kärpänen,*[,1] Caroline A. Heckman,*[,1] Salla Keskitalo,* Michael Jeltsch,* Hanna Ollila,* Gera Neufeld,[§] Luca Tamagnone,[‖] and Kari Alitalo*[,2]

*Molecular/Cancer Biology Laboratory and Ludwig Institute for Cancer Research, Biomedicum Helsinki, Haartman Institute and Helsinki University Central Hospital, University of Helsinki, Helsinki, Finland; [§]Department of Cell Biology and Anatomy, the Rappaport Family Institute for Research in the Medical Sciences, Technion, Israel Institute of Technology, Haifa, Israel; and [‖]Institute for Cancer Research and Treatment (IRCC), University of Turin Medical School, Candiolo, Turin, Italy

ABSTRACT **Lymphatic vascular development is regulated by vascular endothelial growth factor receptor-3 (VEGFR-3), which is activated by its ligands VEGF-C and VEGF-D. Neuropilin-2 (NP2), known to be involved in neuronal development, has also been implicated to play a role in lymphangiogenesis. We aimed to elucidate the mechanism by which NP2 is involved in lymphatic endothelial cell signaling. By *in vitro* binding studies we found that both VEGF-C and VEGF-D interact with NP2, VEGF-C in a heparin-independent and VEGF-D in a heparin-dependent manner. We also mapped the domains of VEGF-C and NP2 required for their binding. The functional importance of the interaction of NP2 with the lymphangiogenic growth factors was demonstrated by cointernalization of NP2 along with VEGFR-3 in endocytic vesicles of lymphatic endothelial cells upon stimulation with VEGF-C or VEGF-D. NP2 also interacted with VEGFR-3 in coprecipitation studies. Our results show that NP2 is directly involved in an active signaling complex with the key regulators of lymphangiogenesis and thus suggest a mechanism by which NP2 functions in the development of the lymphatic vasculature.—Kärpänen, T., Heckman, C. A., Keskitalo, S., Jeltsch, M., Ollila, H., Neufeld, G., Tamagnone, L., Alitalo, K. Functional interaction of VEGF-C and VEGF-D with neuropilin receptors. *FASEB J.* 20, 1462–1472 (2006)**

Key Words: lymphatic endothelial cell · VEGFR-3 · NP2

Early development of the vascular system is dependent on the vascular endothelial growth factor (VEGF) family of ligands and their receptors. Deletion of VEGF receptor-3 (VEGFR-3) is embryonic lethal due to improper remodeling of the primary vascular plexus (1). The formation of lymphatic vessels, which occurs later in development after formation of the cardiovascular system, is also dependent on VEGFR-3 activity (2, 3). Missense mutations of the gene encoding VEGFR-3 that result in impaired signaling from the receptor can lead to hypoplasia of the lymphatic vasculature and to congenital lymphedema (4).

Activation of VEGFR-3 is dependent on binding of its ligands, VEGF-C and VEGF-D. Both factors are angiogenic and lymphangiogenic, and VEGF-C is critical for embryonic development (3, 5, 6). The full-length human VEGF-C and VEGF-D proteins are 48% homologous and share a similar structure with a VEGF homology domain flanked by amino-terminal and carboxy-terminal propetides. Upon secretion, the propeptides are enzymatically cleaved, resulting in the mature signaling molecules with increased affinities to both VEGFR-2 and VEGFR-3 (7, 8).

In addition to VEGF receptors, several VEGF family members also interact with neuropilins. Neuropilins are transmembrane non-tyrosine kinase glycoproteins with a short cytoplasmic domain that has limited signaling capability. In the nervous system, neuropilins mediate axon retraction and guidance by binding class III semaphorins and interacting with members of the plexin receptor family, which act as the signal transducers (9). In the vascular system, neuropilin-1 (NP1) is expressed in arteries (10) whereas NP2 expression is restricted to the lymphatic system and at low levels to veins (11, 12). Along with class III semaphorins, NP1 also binds $VEGF_{165}$, $VEGF\text{-}B_{167}$, $VEGF\text{-}B_{186}$, VEGF-C, and placenta growth factor-2 (PlGF-2) (13–16), whereas NP2 binds $VEGF_{165}$, $VEGF_{145}$, PlGF-2, and VEGF-C (17, 18). NP1 was previously shown to enhance the interaction of $VEGF_{165}$ with VEGFR-2 and to promote endothelial cell proliferation and migration (13,19). While overexpression or deletion of NP1 in mice results in lethal neuronal and vascular defects (20–22), mice deficient for NP2 are viable, displaying mild neuronal abnormalities (23, 24). In addition, NP2 null mice lack small lymphatic vessels and capillaries at birth, while larger lymphatic vessels, along with arteries and veins, remain intact (12). This phenotype suggests that NP2 is

[1] These authors contributed equally to this work.

[2] Correspondence: Molecular/Cancer Biology Laboratory, Biomedicum Helsinki, P.O.B. 63, FI-00014 University of Helsinki, Helsinki, Finland. E-mail: kari.alitalo@helsinki.fi

doi: 10.1096/fj.05-5646com

0892-6638/06/0020-1462 © FASEB

required for proper early development of the lymphatic system, but the underlying molecular mechanisms are still unclear.

For the present studies, we wanted to assess the interaction of neuropilins with lymphangiogenic growth factors and their lymphatic endothelial-specific receptor VEGFR-3. By *in vitro* binding we found that both NP1 and NP2 bind VEGF-C and VEGF-D. We also analyzed the heparin dependency of as well as the domains involved in these interactions. Furthermore, we found that NP2 colocalizes to endocytic vesicles with VEGF-C or VEGF-D after stimulation of primary lymphatic endothelial cells, and observed the interaction of NP2 with VEGFR-3. Together, these results not only indicate that VEGF-C and VEGF-D may in part promote angiogenic signaling by interacting with NP1, but lymphangiogenesis could be modified by the interaction of NP2 with VEGF-C and VEGF-D along with their receptor, VEGFR-3.

MATERIALS AND METHODS

Proteins and antibodies

To produce human VEGF-C in *Drosophila* S2 cells, the pMT-BiP-V5His-C vector was modified to include the hygromycin resistance gene from pCoHygro (both Invitrogen, Carlsbad, CA, USA). Into this resulting vector we cloned VEGF-C nucleotides 658–996 (GenBank accession number X94216) preceded by the melittin signal peptide and followed by a hexahistidine tag. S2 cells were transfected using Effectene (Qiagen, Hilden, Germany). Selection was started 3 days post-transfection with 400 μg/ml hygromycin and the medium was changed every 5 days for 3 wk, after which the supernatant was assayed for protein. For this, the cells were induced for 5 days with 0.5 mM $CuSO_4$, and 15 μl of the conditioned medium was run through 14% SDS-PAGE under reducing conditions. The proteins were identified by Western analysis using both pentahistidine antibody (Ab) (Qiagen) and the specific VEGF-C antiserum 882 (7). For medium scale production, cells were adapted to suspension culture, expanded to 1 l, and induced at a density of 4×10^6 cells/ml. The supernatant was harvested 4.5 days postinduction, cleared by centrifugation, and dialyzed against 50 mM sodium phosphate/300 mM NaCl, pH 6. After dialysis the pH was adjusted to 8.0 by adding 3M Tris/HCl pH 10.5 and the supernatant was centrifuged at 15,000 *g* for 30 min to eliminate precipitate. Batch binding was performed adding 2 ml Ni^{2+}NTA superflow resin (Qiagen), followed by gentle agitation for 12 h at +4°C. The resin was loaded onto a column, washed with 20 mM imidazole, and eluted with a step gradient of 200 mM imidazole. The protein was dialyzed against PBS and sterilized using Millex-GV filters (Millipore, Billerica, MA, USA), then checked on silver-stained reducing SDS-PAGE gels and quantitated using the bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL, USA). Activity was determined using a bioassay as described (2).

Purified human $VEGF_{165}$ and VEGF-D as well as goat polyclonal antibodies against human VEGF-C and VEGF-D were purchased from R&D Systems (Minneapolis, MN, USA). Mouse monoclonal antibodies against human VEGFR-3, 9D9F9 and 2E11D11, were prepared as described (25). Rabbit polyclonal antibodies against NP2 (H-300) and VEGFR-3 (C-20) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), while the rabbit polyclonal antibody for EEA-1 was from Abcam (Cambridge, UK). Rat monoclonal (1121B) and rabbit polyclonal (RS-2) antibodies against VEGFR-2 have been described (26, 27). AlexaFluor488 donkey antimouse, AlexaFluor594 donkey anti-rabbit, and AlexaFluor594 donkey anti-goat secondary antibodies were from Molecular Probes (Eugene, OR, USA).

Cells and cell culture

293T cells were maintained in Dulbecco's modified Eagle's medium (HaartBio, Helsinki, Finland) supplemented with 10% fetal calf serum (PromoCell, Heidelberg, Germany), 2 mM L-glutamine, and 0.2% penicillin/streptomycin sulfate (HaartBio). Human dermal microvascular endothelial cells (HDMVECs) were maintained in endothelial cell medium (both PromoCell) and used at passages 3–7. Porcine aortic endothelial (PAE) cells were kindly provided by Lena Claesson-Welsh (Uppsala University, Sweden) and cultured in F12 medium (HaartBio) supplemented as above.

PAE cells stably expressing VEGFR-3 (R3-PAE) were described previously (28) and maintained with 0.25 μg/ml puromycin (Sigma-Aldrich, St. Louis, MO, USA). To establish PAE and R3-PAE cells stably expressing NP2, the pcDNA3.1z-NP2 plasmid described below was transfected into these cells using JetPEI (Qbiogene, Irvine, CA, USA). Selection antibiotics (NP2-PAE, 200 μg/ml zeocin (Invitrogen); R3/NP2-PAE, 200 μg/ml zeocin and 0.25 μg/ml puromycin) were added to the media 48 h after transfection and the cells grown for 14 days, after which clones were picked. NP2 and VEGFR-3 expression was confirmed by Western analysis.

Expression plasmids

Expression plasmids encoding full-length human VEGF-C (29), human semaphorin 3F (SEMA3F) (30) and full-length human VEGF-D (31) have been described. Expression plasmids coding for VEGF-C mutants were constructed by amplifying the VEGF-C cDNA with the primers 5′-GCGGATCCGACAGAAGAGACTATAAAA-3′ and 5′-GCGGATCCTTAGCTCATTTGTGGTCTTTTCC-3′ for VEGF-CΔN and 5′-GGGATCCGTTCGAGTCCGGACTCG-3′ and 5′-GCGGATCCTTAACGTCTAATAATGGAATG-3′ for VEGF-CΔC. Polymerase chain reaction (PCR) products were cloned in frame with the immunoglobulin (Ig)κ signal sequence to the *Bam*HI site of the pMosaic vector (32). The expression plasmid coding for VEGF-CΔNΔC was constructed by inserting the *Bam*HI-restricted PCR product obtained with oliogonucleotide primers 5′-GGAATTCACAGAAGAGACTATAAA-3′ and 5′-GCGGATCCTTAACGTCTAATAATGGAA-3′ into *Bam*HI/*Eco*RV-opened pAdCMV (29). The resulting vector was opened with *Hind*III, blunted with Klenow enzyme, digested with *Bgl*II, and the sequence coding for the signal peptide of human VEGF-C inserted as an AgeI(blunted)/*Bgl*II fragment obtained from DN VEGF-C (7).

The expression plasmid coding for human NP2 immunoglobulin has been described (18). The cDNA encoding the extracellular domain of human NP1 was assembled by recombinant PCR from Integrated Molecular Analysis of Genomes and their Expression (IMAGE) Consortium cDNA clone 2958475 (Incyte Genomics, St. Louis, MO, USA) containing the sequences coding for the a1a2b1b2 domains and a cDNA fragment coding for the MAM domain obtained by RT-PCR of RNA extracted from HDMVECs with oligonucleotide primers 5′-CCTAGCTAGCCGCAACGATAAATGTGGCGATAC-3′ and 5′-CCTGTGAGCTGGAAGTCATCACCTGTTCCACTGTGGCAGTTGGCCTGGTCGTC-3′ as well as 5′-GTGATGACGACCAGGCCAACTGCCACAGTGGAACAGGTGATGACT-

TCCAGCTCACAG-3′ and 5′-CCTGGATCCGCCAGGTCTGCT-GGTTTTGCAC-3′, respectively, using the underlined primers for the final recombinant PCR. The PCR product was cloned into *Nhe*I/*Bam*HI-cut signal pIgplus vector (Ingenius/R&D Systems) in frame with sequences encoding the CD33 signal peptide and the Fc region of human IgG1. The plasmids coding for the Ig fusion proteins of different neuropilin domains were constructed by PCR amplification of the a1a2 domains with primers *5′-CCTAGCTAGCCGCAACGATAAAT-GTGGCGATAC-3′* and 5′-CCTGGATCCGCGAAATCTTCTG-AGACACTGCTC-3′ for NP1 and 5′-GCCAAGCTTCAACCAGA-CCCACCGTGC-3′ and 5′-CCTAGCGGCCGCTGGCTCTTGG-TGGACCAGG-3′ for NP2; b1b2 domains with primers 5′-GCCAAGCTTTGTATGGAAGCTCTGGGCATG-3′ and 5′-GCGGATCCGCGGCTTCCACTTCACAGCCC-3′ for NP1 and 5′-GCCAAGCTTCTAGAGAACTTTCAGTGCAATG-3′ and 5′-GCGGATCCGCCTTGGAGTCTGTCCAGTCACAG-3′ for NP2; a1a2b1b2 domains with the underlined primers above as well as MAM domains with 5′-GCCAAGCTTGGAACAGGT-GATGACTTCCAG-3′ and 5′-CCTGGATCCGCCAGGTCTG-CTGGTTTTGCAC-3′ for NP1 and 5′-GCCAAGCTTTTTGAG-GATGACAAAGATTTGCA-3′ and 5′-GCGGATCCGC-AAAAGCCGAGATGGGTTCCAT-3′ for NP2. The obtained PCR products were cloned into the signal pIgplus vector as above.

The expression vector encoding the full-length human NP2 a22 isoform (pcDNA3.1z-NP2) was assembled from IMAGE Consortium cDNA clones 1046499, 1564852, and 2728688 as well as the pcDNA3.1z+ vector (Invitrogen) by restriction digest and recombinant PCR. The expression vector encoding the full-length human VEGFR-3 was constructed from the one already described (33) by replacing the pcDNA3.1z+ backbone with pcDNA3.1hygro (both Invitrogen).

In vitro binding assays

293T cells were transiently transfected with plasmids encoding full-length VEGF-C, full-length VEGF-D, VEGF-CΔNΔC, VEGF-CΔN, VEGF-CΔC, or $VEGF_{165}$, or with an empty plasmid using JetPEI reagent. After 48 h the cells were metabolically labeled with 100 μCi/ml [^{35}S]-methionine and [^{35}S]-cysteine (Redivue ProMix, Amersham Biosciences, Uppsala, Sweden) in cysteine and methionine-free MEM (HaartBio) overnight. For binding assays, the conditioned media were supplemented with 0.5% BSA and 0.02% Tween 20, with or without addition of 10 μg/ml heparin (Gibco BRL/Invitrogen). Labeled proteins were bound with 200 ng of VEGFR-2 Ig (34) or VEGFR-3 immunoglobulin (2) or with conditioned media from 293T cells transiently transfected to express NP1 Ig or NP2 Ig fusion proteins. Protein complexes were precipitated with protein A-Sepharose beads (Amersham Biosciences) and washed three times with PBS supplemented with 0.5% BSA and 0.02% Tween 20, and once with PBS. Bound proteins were separated by 12% SDS-PAGE under reducing conditions and analyzed by autoradiography.

For binding competition assays, metabolically labeled conditioned medium from 293T cells transfected with the full-length VEGF-C construct was mixed with increasing amounts of unlabeled conditioned medium from 293T cells transfected with SEMA3F expression vector. The conditioned media were supplemented with 0.5% BSA, 0.02% Tween 20, 10 μg/ml heparin and bound to NP1 Ig or NP2 Ig fusion proteins. Protein complexes were analyzed as above.

Coprecipitation assays

293T cells were transiently transfected with pcDNAhygro-VEGFR-3 and pcDNAz-NP2 and cultured for 48 h. Lymphatic and blood vascular endothelial cells (LECs and BECs, respectively) were separated from mixed HDMVEC cultures as described previously (35) and grown to confluency on fibronectin (Sigma-Aldrich) -coated plates. Transfected 293T cells, LECs and BECs were serum-starved overnight and stimulated with 100 ng/ml VEGF-C for 10 min at 37°C. After stimulation the cells were rinsed with cold PBS and lysed on ice in PLCLB buffer (50 mM HEPES, pH 7.5, 1% Triton X-100, 5% glycerol, 1 mM EGTA, 150 mM NaCl, 1.5 mM $MgCl_2$, 100 mM NaF) containing protease inhibitors (0.5 mM PMSF, 15 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM Na_3VO_4). Equal amounts of protein were immunoprecipitated with anti-VEGFR-3 9D9F9 (3 μg/sample; 293T) or C-20 (1 μg/sample; LECs), 1121B (3 μg/sample) and anti-NP2 H-300 (1 μg/sample) antibodies in the presence of 0.5% BSA, 0.02% Tween 20 and pulled down with protein G- (9D9F9; 1121B) or protein A- (H-300; C-20) Sepharose. Sepharose beads were washed three times with binding buffer and once with PBS. Samples were electrophorized on 7.5% SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes (Schleicher & Schuell Bioscience GmbH, Dassel, Germany). Immunoprecipitated proteins from 293T cells were detected with 9D9F9 and H-300 primary and horseradish peroxidase (HRP) -conjugated secondary antibodies (DakoCytomation, Glostrup, Denmark), and proteins from LEC and BEC lysates with C-20 or RS-2 and H-300 primary antibodies followed by biotinylated anti-rabbit Ab (DakoCytomation) and streptavidin-biotinylated HRP-conjugate (Amersham Biosciencies). Signals were visualized by chemoluminescence using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology).

Cell stimulations and immunofluorescent staining

Approximately 50,000 HDMVECs or PAE cells were seeded in 24-well plates onto glass coverslips. For HDMVECs the coverslips were pretreated with 5 μg/ml fibronectin in PBS. When nearly confluent, the cells were serum starved overnight, stimulated with 100 ng/ml VEGF-C, 500 ng/ml VEGF-D, or 100 ng/ml $VEGF_{165}$ for 15–30 min at 37°C, and fixed for 10 min at room temperature with 4% paraformaldehyde in PBS. The coverslips were washed three times with PBS for 5 min each, after which the cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min. After blocking with 2% BSA and 0.1% Triton X-100 in PBS for 20–60 min, the coverslips were incubated with primary antibodies in blocking buffer (40 μg/ml 2E11D11 (HDMECs) or 7.4 μg/ml 9D9F9 (PAE cells) anti-VEGFR-3, 2 μg/ml anti-NP2, 5 μg/ml anti-EEA-1, 2 μg/ml anti-VEGF-C, or 2 μg/ml anti-VEGF-D) for 1 h and rinsed three times for 5 min with PBS. This was followed by incubation with secondary antibodies (6.6 μg/ml each) in blocking buffer for 1 h. After washing, the coverslips were mounted on glass slides with VectaShield containing 4′,6′-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA) and examined using the Zeiss LSM 510 Meta confocal microscope. 405, 488 and 543 nM laser lines were used to analyze for DAPI, AlexaFluor488 and AlexaFluor594, respectively. All images were taken with a 63 × Plan-Apochromat objective.

RESULTS

VEGF-C and VEGF-D bind to both neuropilins

To assess the interaction of VEGF-C and VEGF-D with neuropilins, we used conditioned media from cells

transfected with plasmids encoding full-length VEGF-C or VEGF-D and soluble Ig fusion proteins of NP1 or NP2 in *in vitro* binding assays. As shown in **Fig. 1*B*** (top panel), both partially processed (29/31 kDa, arrow) and mature (21 kDa, arrowhead) forms of VEGF-C interacted with NP2, and these interactions were slightly enhanced by heparin. We also observed the binding of partially processed VEGF-C to NP1, but this was dependent on the presence of heparin (Fig. 1*B*). Both NP1 and NP2 bound VEGF-D, but these interactions were limited to the partially processed form of this growth factor and were strictly heparin dependent (Fig. 1*B*, middle panel, arrow).

VEGF-C domain interaction with neuropilins

To characterize the VEGF-C domains involved in neuropilin binding, we employed expression plasmids coding for VEGF-C lacking the amino-terminal (VEGF-CΔN) or carboxy-terminal (VEGF-CΔC) propeptide or both propeptides (VEGF-CΔNΔC) (schematic representation in Fig. 1*A*). As expected, VEGF-CΔNΔC does not bind to NP1 whether in the presence or absence of heparin, but binds to NP2 independent of heparin (Fig. 1*C*, top panel, arrowheads). Whereas the deletion of the carboxy-terminus of VEGF-C does not significantly affect the neuropilin binding properties of VEGF-C, deletion of the amino-terminus abolishes NP1 binding and leads to a dramatic reduction of binding to NP2 (Fig. 1*C*, middle and bottom panels). These results suggest that although the mature form of VEGF-C alone is capable of binding to NP2, the amino-terminus of VEGF-C greatly enhances its interaction with NP2 and is the major mediator of NP1 binding. Deletion of the VEGF-C carboxy-terminus leads to two likely differentially glycosylated or alternatively processed forms of VEGF-C (Fig. 1*C*, lower panel).

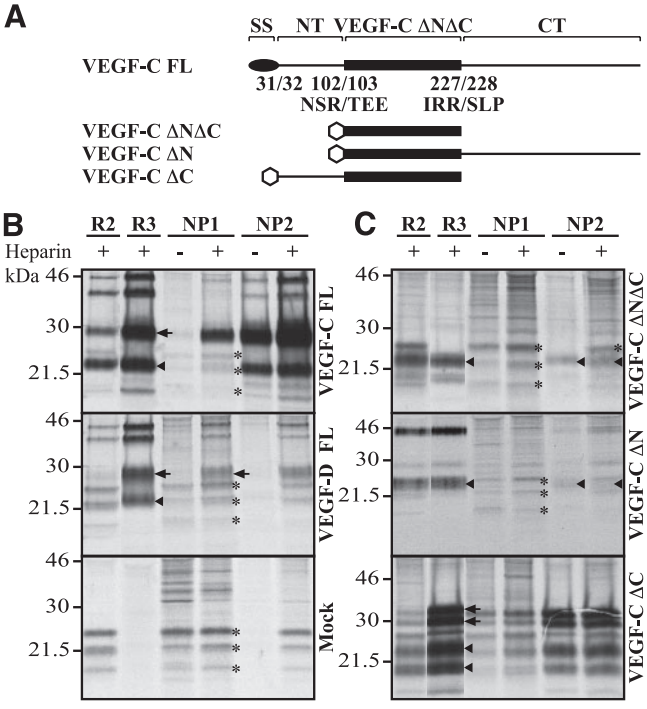

Figure 1. VEGF-C and VEGF-D bind to neuropilin-1 and -2. *A)* Schematic presentation of various VEGF-C constructs. Box, mature fully processed form of VEGF-C (VEGF-CΔNΔC); line, amino- (NT) and carboxy-terminal CT domains of VEGF-C; oval, endogenous signal sequence of VEGF-C (SS); hexagon, heterologous signal sequence of the Igκ chain. *B, C)* Binding of metabolically labeled conditioned media from VEGF-C FL, VEGF-D FL and mock (*B*) as well as VEGF-CΔNΔC, VEGF-CΔN, and VEGF-CΔC (*C*) transfected 293T cells to soluble VEGFR-2 (R2) and VEGFR-3 (R3) as well as to neuropilin-1 (NP1) and neuropilin-2 (NP2) Ig fusion proteins with or without addition of heparin as indicated. Protein complexes were precipitated with protein A-Sepharose, separated under reducing conditions by 12% SDS-PAGE, and autoradiographed. Bands corresponding to endogenous VEGF are marked with asterisks, the ones corresponding to the mature forms of VEGF-C and VEGF-D with arrowheads and those corresponding to intermediately processed forms with arrows.

Neuropilins bind VEGF-C through their b1b2 domains

Neuropilins consist of two a domains (CUB domains), two b domains (coagulation factor V/VIII homology domains), and a c domain (MAM domain), followed by a transmembrane and a short intracellular domain (schematic representation in **Fig. 2*A***). It was reported that NP1 binds $VEGF_{165}$ and PlGF-2 as well as heparin through its b1b2 domains, whereas neither of these domains alone nor the b1 domain in combination with the a1a2 domains is able to bind $VEGF_{165}$ (36,37). To determine which domains of NP1 and NP2 are responsible for VEGF-C binding, we made expression constructs coding for soluble Ig fusion proteins of the a1a2, b1b2, a1a2b1b2, and c domains of NP1 and NP2 (schematic presentation in Fig. 2*A*). All constructs produced Ig fusion proteins of expected size and approximately equal expression levels, as did constructs coding for the Ig fusion proteins containing the entire extracellular domains of the two receptors (Fig. 2*B*). The b1b2 domains of both NP1 and NP2 were sufficient to bind VEGF-C (Fig. 2*C*, upper panels). VEGF-C binding to NP1, either to the whole extracellular domain or to the b1b2 or a1a2b1b2 domains, was dependent on heparin (Fig. 2*C*, upper left panel). On the contrary, whereas binding of VEGF-C to the b1b2 domains of NP2 was dependent on heparin, supplementation with the other neuropilin domains abolished the requirement for heparin, but its addition still slightly increased the binding (Fig. 2*C*, upper right panel). In agreement with previous publications, the b1b2 domains of NP1 were sufficient for binding to $VEGF_{165}$, and the binding was slightly increased by addition of 10 μg/ml heparin (Fig. 2*C*, lower left panel). Binding of $VEGF_{165}$ to NP2, on the contrary, depended strictly on the presence of heparin (Fig. 2*C*, lower right panel).

Neuropilin interaction with class III semaphorins, including semaphorin 3F (SEMA3F), is partially mediated by their b1b2 domains (37–39). Using increasing

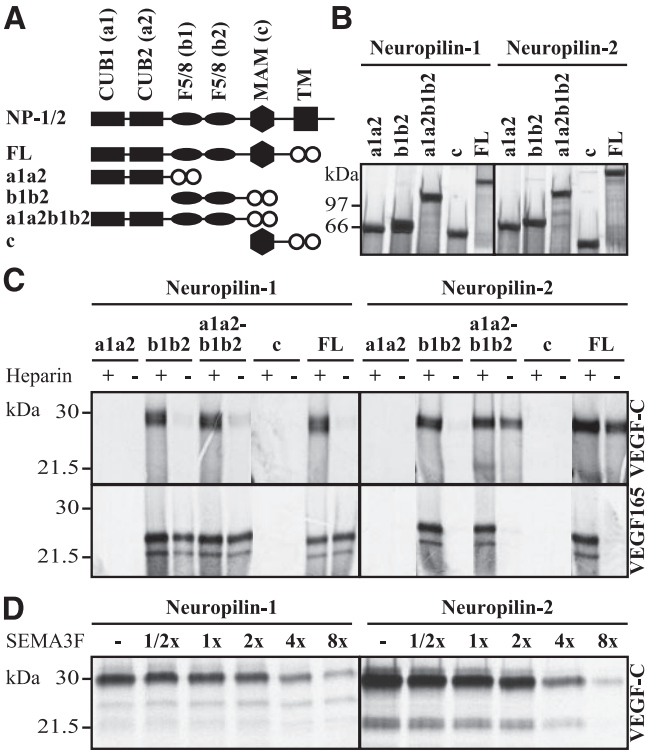

Figure 2. Neuropilins bind VEGF-C through their b1b2 domains. *A)* Schematic presentation of the structure of NP1 and NP2 as well as the soluble Ig fusion proteins of the different neuropilin domains. Box, CUB domain (a); oval, coagulation factor V/VIII (F5/8) homology domain (b); hexagon, MAM domain (c), square, transmembrane domain (TM); open double circle, IgG Fc domain. *B)* Expression of soluble Ig fusion proteins of different NP1 and NP2 domains. Expression contructs were transfected into 293T cells, the cells metabolically labeled, and Ig fusion proteins precipitated from the conditioned media with protein A-Sepharose. *C)* Binding of VEGF-C and $VEGF_{165}$ to the different NP1 and NP2 domain Ig fusion proteins with and without heparin as indicated. Conditioned media from unlabeled 293T cells transfected with expression constructs coding for soluble Ig fusion proteins of different NP1 and NP2 domains were used to precipitate metabolically labeled VEGF-C or $VEGF_{165}$ from conditioned media of transfected 293T cells. *D)* Competition of increasing amounts of unlabeled SEMA3F with metabolically labeled VEGF-C for binding to NP1-Ig and NP2-Ig fusion proteins. Bound proteins were analyzed by SDS-PAGE on 7.5% (*B*) or 12% (*C, D*) gels and autoradiographed.

amounts of unlabeled conditioned media from cells transfected with a SEMA3F expression vector, we found that SEMA3F could compete with metabolically labeled VEGF-C for the binding to NP1-Ig and NP2-Ig fusion proteins (Fig. 2*D*). This suggests that SEMA3F and VEGF-C interact with neuropilins through overlapping binding sites.

NP2 colocalizes with VEGF-C and VEGF-D in stimulated lymphatic endothelial cells

HDMVECs include both blood and lymphatic endothelial cells (BECs and LECs, respectively). When maintained in serum-free conditions, HDMVECs display expression of VEGFR-3 and NP2 on the plasma membrane (**Fig. 3*A***). Expression of VEGFR-3 is significantly higher on LECs than BECs (Fig. 3*A*), and can be used as a marker to distinguish the two cell types (35). Cell surface expression of NP2 also appeared slightly higher on LECs than BECs (Fig. 3*A*). To provide evidence of the interaction between lymphangiogenic ligands and NP2 in a cellular context, HDMVECs were stimulated with VEGF-C or VEGF-D, then fixed and stained. Receptors are often internalized upon ligand binding, after which they are recycled to the cell surface or transported to the lysosomal compartment and de-

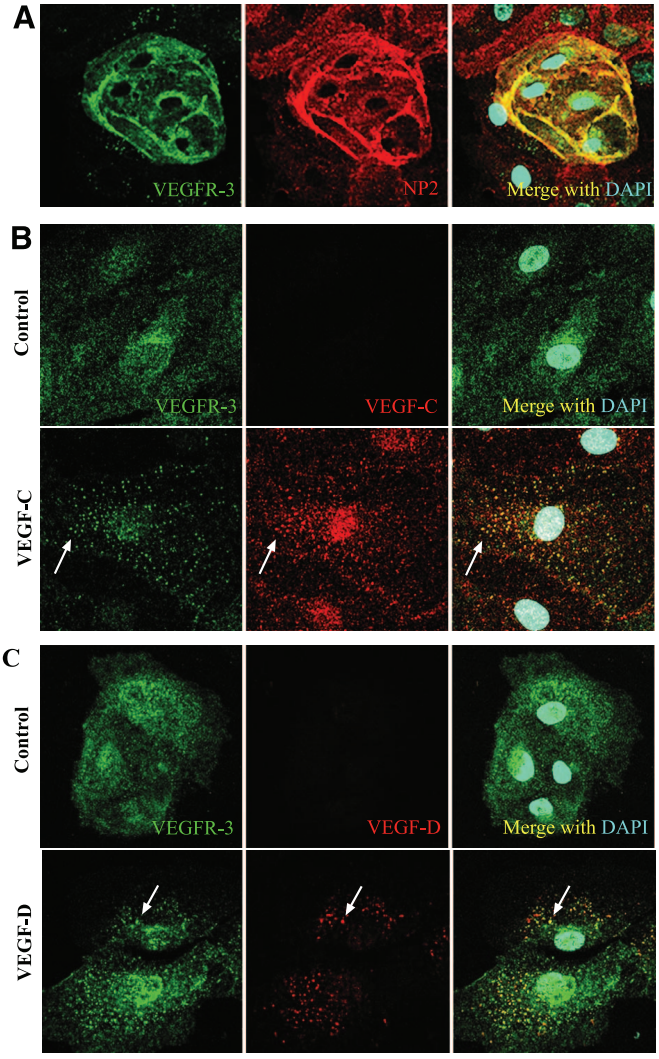

Figure 3. Exogenous VEGF-C and VEGF-D colocalize with NP2 in lymphatic endothelial cells. *A)* Immunofluorescence analysis of VEGFR-3 (green) and NP2 (red) expression on the surface of HDMVECs. Blocking and antibody incubations were carried out without Triton X-100. The far right panel represents the merged images along with the cell-permeant DAPI nuclear dye signal (light blue). *B, C)* HDMVECs were stimulated with control protein, VEGF-C or VEGF-D for 15 min then fixed, permeabilized and stained for NP2 (green) and VEGF-C (red) or VEGF-D (red). The far right panels are the merged signals with DAPI. White arrows indicate examples of internalized receptor and ligand. These experiments were performed at least three times.

graded. Visualization of receptor internalization therefore provides a read-out of ligand receptor signaling activity. As shown in Fig. 3*B* and *C*, VEGF-C and -D were internalized and colocalized with NP2 to endocytic vesicles, suggesting that the interaction of these ligands with NP2 is maintained within the cell and composes an active signaling complex.

Interaction of NP2 and VEGFR-3

Neuropilins have a short cytoplasmic domain, and signaling largely occurs through associations with other receptors. We therefore wanted to determine whether an interaction occurs between NP2 and VEGFR-3. For these studies we used transfected 293T cells and purified LEC cultures. After transfection with expression vectors encoding the two receptors, 293T cells were stimulated with VEGF-C or control protein, then lysed. Immunoprecipitation followed by Western analysis showed a ligand-independent interaction of NP2 with VEGFR-3 in transfected cells (**Fig. 4*A***). Interaction between NP2 and VEGFR-3 was also observed in isolated LEC cultures endogenously expressing these two receptors, as shown by immunoprecipitation for either NP2 or VEGFR-3 followed by Western blot (Fig. 4*B*). On the other hand, we were unable to detect coprecipitation of NP2 with VEGFR-2 from lysates of isolated LEC or BEC cultures (Fig. 4*C* and data not shown).

VEGF-C- and VEGF-D-induced internalization of VEGFR-3 and NP2

To verify results from the immunoprecipitation studies and further assess for an association between endogenous NP2 and VEGFR-3 in endothelial cells, HDMVECs were stimulated with different growth factors, then fixed and stained for the two receptors. VEGFR-3 and NP2 were internalized and colocalized to endocytic structures upon stimulation with VEGF-C or VEGF-D (**Fig. 5*B, C***). Although we observed the interaction of $VEGF_{165}$ with NP2 by *in vitro* binding assay, there was no clear change in NP2 localization after stimulation of LECs with that ligand (Fig. 5*D*). VEGFR-3 also colocalized with the early endosome marker EEA-1 in VEGF-C or VEGF-D stimulated HDMVECs, confirming that the receptors are indeed internalized to endocytic vesicles and not clustered on the cell surface (**Fig. 6*A–C***). These results indicate that, in LECs, an association is maintained between NP2 and VEGFR-3 after binding of lymphangiogenic ligands.

VEGF-C-induced internalization of NP2 and VEGFR-3 is dependent on VEGFR-3

Activation of cell signaling often coincides with receptor-mediated endocytosis (40). Our observations with LECs suggested that this process was also involved in VEGF-C and VEGF-D mediated signaling through VEGFR-3 and NP2. Furthermore, this mechanism was recently shown to coordinate cell migration by the

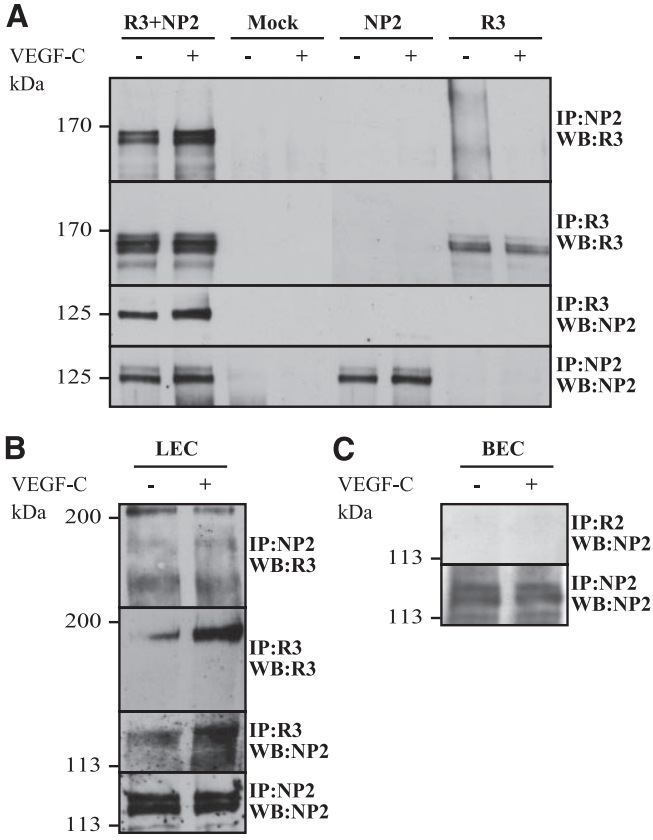

Figure 4. NP2 and VEGFR-3 coprecipitate. *A)* 293T cells were transiently transfected with expression vectors encoding VEGFR-3 and NP2, an empty vector, or the NP2 or VEGFR-3 expression vectors alone. Transfections were performed in duplicate. Cells were stimulated with VEGF-C (+) or control protein (–) for 10 min, then lysed. Equal amounts of protein from cell lysates were immunoprecipitated using antibodies against VEGFR-3 or NP2, then separated by 7.5% SDS-PAGE under reducing conditions and transferred to nitrocellulose. Western analysis was performed with anti-VEGFR-3 or NP2 antibodies as indicated. *B, C)* Purified LEC or BEC cultures were stimulated with VEGF-C (+) or control protein (–) and lysed. Equal amounts of protein were subjected to immunoprecipitation using antibodies against NP2 and either VEGFR-3 (*B*) or VEGFR-2 (*C*) and Western analysis as described above. The three VEGFR-3 bands represent the full-length, an intracellular, nonglycosylated precursor, and the proteolytically cleaved forms of VEGFR-3.

intracellular localization of receptor tyrosine kinase activity (41). As NP1 can enhance $VEGF_{165}$-induced endothelial cell migration (13, 42), we hypothesized that NP2 may have a similar function in part by modulating ligand-induced VEGFR-3 endocytosis. To determine if internalization is dependent on one or the other receptors, we used PAE cells expressing either VEGFR-3 or NP2 or both receptors together. While VEGF-C induced the internalization of VEGFR-3 in PAE cells expressing that receptor alone (**Fig. 7*A***), VEGF-C did not induce internalization of NP2 in the NP2-expressing PAE cells (Fig. 7*B*). However, NP2 was internalized when expressed together with VEGFR-3 (Fig. 7*C*). These results indicate that VEGF-C-induced receptor endocytosis is dependent on VEGFR-3 rather than on NP2.

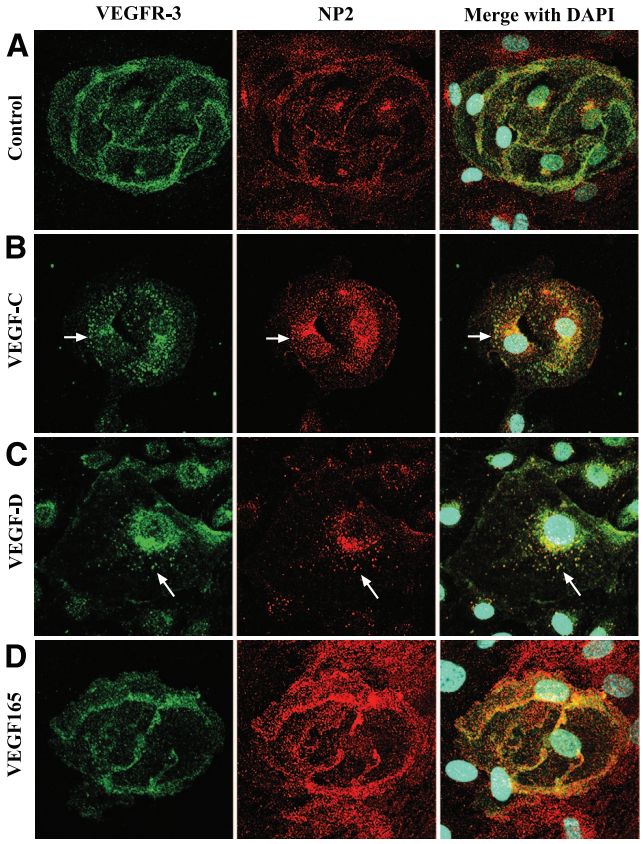

Figure 5. VEGF-C- and VEGF-D-induced internalization of VEGFR-3 and NP2. HDMVECs were stimulated with control protein (*A*), VEGF-C (*B*), VEGF-D (*C*), or $VEGF_{165}$ (*D*) for 15 min then fixed, permeabilized, and stained for VEGFR-3 (green) and NP2 (red). The far right panels represent the merged images along with the DAPI nuclear stain (light blue). White arrows indicate examples of internalized receptors. These are representative of at least 3 experiments.

DISCUSSION

Although several studies have shown the importance of NP1 for angiogenesis, there is little information concerning the significance of neuropilin receptors for lymphatic vessel development, particularly at the molecular and cellular levels. In this study we analyzed the interaction of neuropilins with lymphangiogenic factors. While VEGF-C itself is sufficient to bind to NP2, the interaction of VEGF-C with NP1 and that of VEGF-D with both neuropilins requires heparin. Although the fully processed form of VEGF-C can bind to NP2, this interaction is greatly enhanced by the amino-terminus. Domain analysis of NP2 revealed that the b1b2 domains are sufficient to bind VEGF-C, but only in the presence of heparin, whereas the addition of the a1a2 domains allows for a heparin-independent interaction. Moreover, we showed that the class III semaphorin SEMA3F competes with VEGF-C for binding to NP1 and NP2, suggesting that the two ligands have overlapping binding sites in these receptors. NP2 was shown to coprecipitate with VEGFR-3, likely indicating a cooperative signaling pathway where NP2 acts as a coreceptor for VEGFR-3 to modulate its lymphangiogenic function. This possibility was further strengthened by analysis of human lymphatic endothelial cells, in which we observed the cointernalization and colocalization of NP2 and VEGFR-3 after stimulation with VEGF-C or VEGF-D. However, VEGF-C- and VEGF-D-induced signaling was dependent on VEGFR-3 rather than NP2, as demonstrated by receptor localization changes after ligand stimulation of LECs and VEGFR-3 and NP2 expressing PAE cells.

In the vascular system, neuropilins act as receptors for both VEGFs and semaphorins. Deletion of NP1 in mice is embryonic lethal and results in severe neuronal and vascular abnormalities (21, 22). The vascular defects, which include impaired vascularization of the central and peripheral nervous systems and improper development and organization of the branchial arches and great vessels, were originally believed to be due to the loss of VEGF rather than semaphorin signaling through NP1 (22). However, disruption of semaphorin signaling may add to these abnormalities as well (43, 44). Death of $NP1^{-/-}$ embryos occurs prior to formation of the lymphatic vascular system, at the stage when VEGFR-3 is still expressed in and required for development of the blood vascular system. VEGF-C and VEGF-D are angiogenic as well as lymphangiogenic (5, 6), signaling through VEGFR-2 and VEGFR-3 (7, 8, 45). The fact that both growth factors bind NP1 may indicate that NP1 modulates the angiogenic function of these ligands.

In contrast, mice deficient for NP2 are viable, exhibiting only mild defects in the nervous and lymphatic

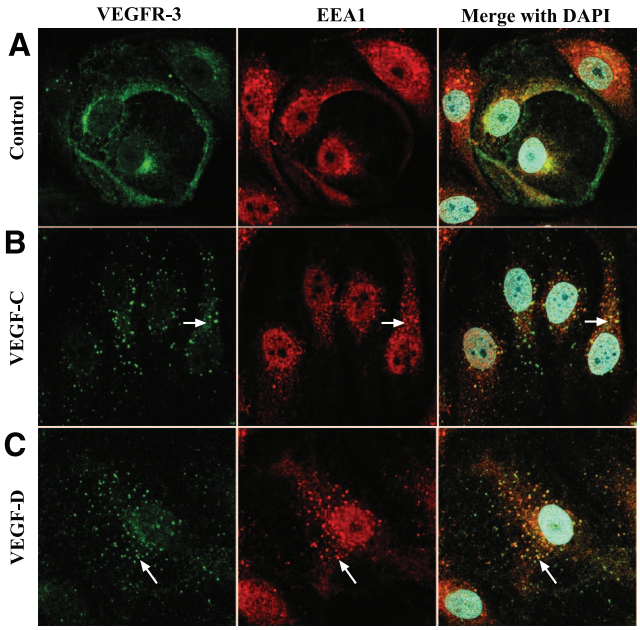

Figure 6. VEGFR-3 colocalizes with the early endosome marker EEA-1. HDMVECs were stimulated with control protein (*A*), VEGF-C (*B*), or VEGF-D (*C*) for 15 min then fixed, permeabilized, and stained for VEGFR-3 (green) and EEA-1 (red). Panels on the far right side show merged images with the DAPI nuclear stain. White arrows indicate colocalization of VEGFR-3 and EEA-1 signals. These are representative of at least 2 experiments.

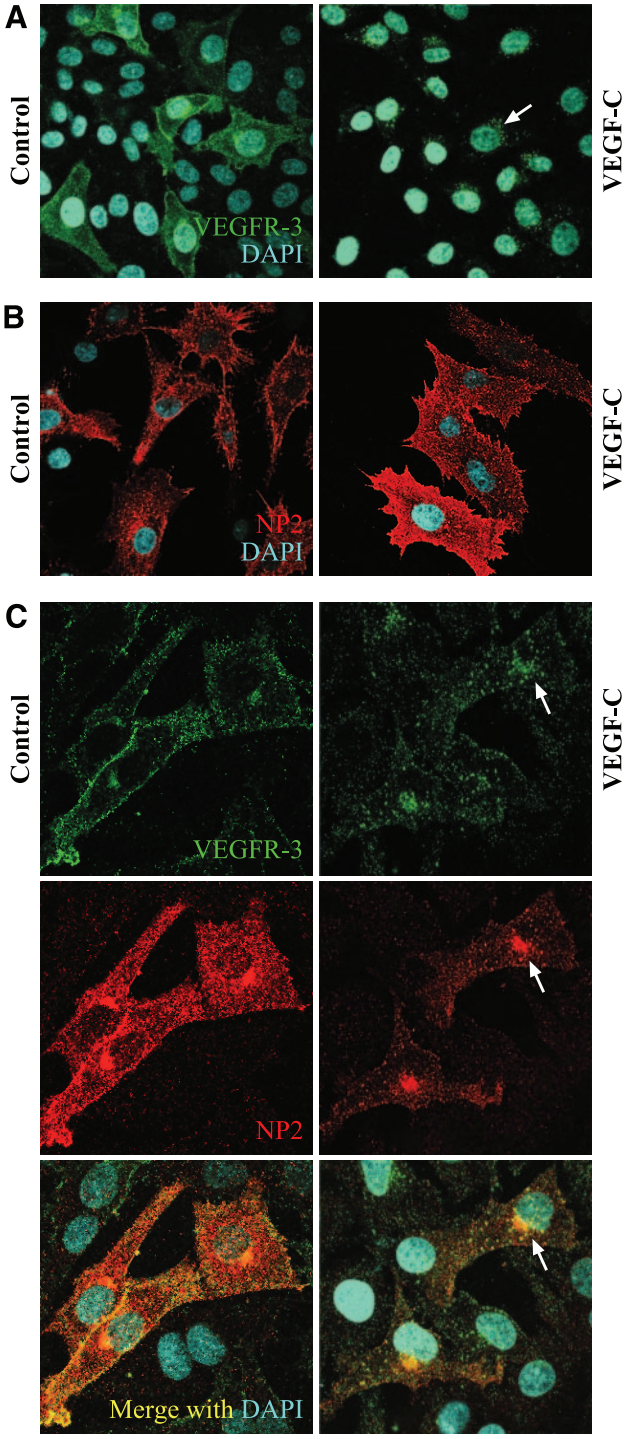

Figure 7. VEGF-C-induced receptor internalization is VEGFR-3 dependent. *A)* PAE cells stably expressing human VEGFR-3 were stimulated with control protein or VEGF-C for 30 min then fixed, permeabilized, stained for VEGFR-3 (green), and mounted with DAPI for nuclear staining (light blue). *B)* A similar assay was performed on PAE cells stably expressing human NP2 and the cells stained with anti-NP2 antibodies (red). *C)* PAE cells stably expressing human VEGFR-3 and NP2 were stimulated with control protein or VEGF-C, and analyzed as in panels *A*, and *B*. White arrows indicate signals from internalized receptors. These experiments were performed at least twice.

vascular systems (12, 23, 24). The lymphatic defect, which is the absence of small lymphatic capillaries at birth, is normalized during the first postnatal week (12). This suggests that NP2 is required for lymphatic-specific signals during embryonic development and the early postnatal period. Although it remains to be determined whether semaphorin signaling through NP2 is functionally significant for lymphangiogenesis, our results suggest that the lymphangiogenic potential of NP2 is likely dependent on its ability to bind VEGF-C and VEGF-D and to interact with VEGFR-3.

Heparan sulfate proteoglycans are a constituent of the extracellular matrix and function to sequester heparin binding growth factors, thereby altering ligand availability to receptors and creating ligand concentration gradients. Cell surface heparan sulfates are necessary for the formation of complexes between basic fibroblast growth factor (bFGF) and FGFR, and greatly facilitate the binding of several other growth factors to their cell surface high-affinity receptors (46, 47). It has also been suggested that heparin greatly enhances the binding of VEGF to VEGFR-2 and to neuropilins (36, 48). In addition, the b1b2 domains of NP1 directly bind heparin (36). Our results suggest that interaction with cell surface heparan sulfate proteoglycans is also involved in the binding of VEGF-C and VEGF-D to neuropilins. Although VEGF-C is able to bind NP2 without heparin, addition of heparin slightly enhances this interaction and is even more important for the binding of VEGF-C to NP-1 and of VEGF-D to both neuropilins.

Domain analysis of NP1 and NP2 showed that binding of VEGF-C occurred through the b1b2 domains of both receptors, and these domains alone could interact with the growth factor in the presence of heparin. Addition of the a1a2 domains of NP2 resulted in a heparin-independent interaction. Perhaps the a1a2 domains or heparin are needed for charge interactions with the ligand or for altering receptor conformation so that ligand accessibility and avidity are increased. In the presence of heparin, the MW of NP1 is increased, suggesting that heparin induces multimerization of the receptor (49). The b1b2 domains of neuropilins are also involved in the binding of class III semaphorins (37–39). We found that SEMA3F could compete with VEGF-C for binding to either NP1 or NP2, indicating that these different ligands have overlapping binding sites on both receptors. However, VEGF-C could not compete for SEMA3F binding to NP1 or NP2 (data not shown), suggesting that the affinity of SEMA3F for these receptors is greater than that of VEGF-C.

Upon secretion, the carboxy-terminal propeptide of the VEGF-C precursor is proteolytically cleaved but remains covalently attached to the amino-terminal propeptide (7). After cleavage of the amino-terminus, both propeptides are released, leaving the mature fully processed VEGF-C peptide. Proteolytic processing controls the receptor affinity and specificity of VEGF-C and VEGF-D in that upon proteolytic cleavage, the affinity of these growth factors toward VEGFR-3 increases and

the fully processed mature forms of VEGF-C and human VEGF-D bind to and also activate VEGFR-2 (7, 8, 45). The different processed forms of VEGF-C also appeared to vary in their affinity for neuropilins. Although the mature form of VEGF-C binds to NP2, our results suggest that the amino-terminus is required for optimal interaction. These data indicate that neuropilins would preferentially interact with the unprocessed or semiprocessed form of VEGF-C. It is possible that neuropilins are used to sense the secreted immature forms of the ligand. After binding, VEGF-C would undergo further proteolytic cleavage, thereby increasing interactions with VEGFR-2 and VEGFR-3. On the other hand, the different processed forms of VEGF-C may act in a similar manner as the different splice isoforms of VEGF, which bind specific receptors and have unique biological functions (50). In this case, neuropilins would primarily modulate the response of cells to immature VEGF-C but have little effect on the cellular response to mature VEGF-C.

The mechanism used by NP2 to convey VEGF-C- and VEGF-D-induced signals likely involves the interaction of NP2 with VEGFR-3. Our data from the immunoprecipitation studies suggests that the receptors are capable of interacting. In addition, endogenous receptors colocalized to endocytic vesicles after stimulation of HDMVECs with VEGF-C or VEGF-D, further indicating a close relationship between NP2 and VEGFR-3. We did not observe NP2 endocytosis when cells were stimulated with $VEGF_{165}$, although our results and previous studies have shown that this ligand can bind to NP2 (17). As $VEGF_{165}$ induces the internalization of VEGFR-2 and its subsequent degradation (51), it is plausible that NP2 would also be internalized if it acts as a coreceptor for VEGFR-2. However, at least in lymphatic endothelial cells, this did not occur and we were unable to observe coprecipitation of NP2 along with VEGFR-2 in both LECs and BECs. If NP2 was significant in such a complex and could modulate VEGFR-2 signaling, the loss of NP2 would likely lead to severe vascular abnormalities during development. Yet the vascular defect in $NP2^{-/-}$ mice is confined to the loss of small lymphatic capillaries and to a reduction of VEGF-induced retinal neovascularization (52); therefore, it seems unlikely that NP2 has a major contribution in $VEGF_{165}$ and VEGFR-2 signaling in the vascular system. While NP1 is involved in VEGFR-2 signaling in the blood vascular system, NP2 may be needed primarily for the lymphangiogenic signaling of VEGF-C and VEGF-D through VEGFR-3. Nevertheless, the possibility of complex formation between NP2 and VEGFR-2 or NP1 and VEGFR-3 upon VEGF-C and VEGF-D binding cannot be excluded.

Endocytosis of active receptor ligand complexes can serve a number of purposes in modifying the signal output. Originally, this mechanism was thought to down-regulate signaling, as internalized receptors are often targeted for degradation. However, additional studies have shown that endocytosis of the TGF-β receptor complex directs the active receptor to downstream internal signal mediators (53). More recently it was shown that internal localization of the tyrosine kinase receptors EGFR and PVR is important for maintaining signal polarity, which is necessary for directed cell migration (41). Given that the primary function of neuropilins is to act as receptors for guidance molecules, it was reasonable to hypothesize that these receptors may serve to modulate VEGF signaling by directing active VEGFRs within the cell. As a read-out of receptor signaling, we visualized receptor internalization. Localization of NP2 expressed alone in PAE cells remain unchanged after stimulation with ligand while VEGFR-3 could be internalized without NP2. Although these results indicated that NP2 is not necessary for the endocytic process, it is possible that neuropilins may function to specifically localize VEGFR signaling or to modify the cellular response by as of yet unknown mechanisms. Indeed, we have observed that the chemotactic response of LECs to full-length VEGF-C was significantly reduced when cells were pretreated with a blocking NP2 Ab, indicating the functional importance of NP2 for lymphatic endothelial cell migration (S. Keskitalo and C. Heckman, unpublished data).

Neuropilins are capable of interacting with a number of different ligands, and we have now shown how both NP1 and NP2 bind the lymphangiogenic ligands VEGF-C and VEGF-D. The ability of NP2 to bind these ligands and interact with VEGFR-3 provides a likely explanation for the lymphatic vascular defect observed in NP2 null mice. Whether neuropilins act purely as accessory receptors seeking out and binding signaling molecules such as semaphorins and VEGFs, or if they have additional roles such as localizing receptor activity or ensuring transduction of particular signals from coreceptors, remains to be determined. However, these data add to the versatility of neuropilins expanding their significance in lymphatic vessel development. FJ

We gratefully acknowledge the excellent technical assistance of Mari Helanterä, Paula Hyvärinen, Tanja Laakkonen, Sanna Lampi, and Tapio Tainola. We thank Dr. Lena Claesson-Welsh (Uppsala University, Uppsala, Sweden) for providing the PAE cells and the RS-2 Ab, Dr. Marc G. Achen and Steven A. Stocker (Ludwig Institute for Cancer Research, Melbourne, Australia) for the VEGF-D cDNA and Dr. Marika J. Kärkkäinen for sharing preliminary data. The 1121B Ab was a kind gift from Dr. Bronek Pytowski (ImClone Systems). This study was supported by grants from the European Union (Lymphangiogenomics LSHG-CT-2004–503573), NIH (5 R01 HL075183–02), the Academy of Finland (202852 and 204312 K.A.; 207803 C.A.H.), the Novo Nordisk Foundation, Finnish Cancer Organizations, the Science Foundation of Instrumentarium, Finnish Cultural Foundation, Finnish Heart Association, the Paulo Foundation, K.A. Johanssons Foundation, Biomedicum Helsinki Foundation and Helsinki Biomedical Graduate School (S.K.).

REFERENCES

1. Dumont, D. J., Jussila, L., Taipale, J., Lymboussaki, A., Mustonen, T., Pajusola, K., Breitman, M., and Alitalo, K. (1998)

Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. *Science* **282,** 946–949
2. Makinen, T., Jussila, L., Veikkola, T., Karpanen, T., Kettunen, M. I., Pulkkanen, K. J., Kauppinen, R., Jackson, D. G., Kubo, H., Nishikawa, S., Yla-Herttuala, S., and Alitalo, K. (2001) Inhibition of lymphangiogenesis with resulting lymphedema in transgenic mice expressing soluble VEGF receptor-3. *Nat. Med.* **7,** 199–205
3. Karkkainen, M. J., Haiko, P., Sainio, K., Partanen, J., Taipale, J., Petrova, T. V., Jeltsch, M., Jackson, D. G., Talikka, M., Rauvala, H., Betsholtz, C., and Alitalo, K. (2004) Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. *Nat. Immunol.* **5,** 74–80
4. Karkkainen, M. J., Ferrell, R. E., Lawrence, E. C., Kimak, M. A., Levinson, K. L., McTigue, M. A., Alitalo, K., and Finegold, D. N. (2000) Missense mutations interfere with VEGFR-3 signalling in primary lymphoedema. *Nat. Genet.* **25,** 153–159
5. Witzenbichler, B., Asahara, T., Murohara, T., Silver, M., Spyridopoulos, I., Magner, M., Principe, N., Kearney, M., Hu, J.-S., and Isner, J. M. (1998) Vascular endothelial growth factor-C (VEGF-C/VEGF-2) promotes angiogenesis in the setting of tissue ischemia. *Am. J. Pathol.* **153,** 381–394
6. Jeltsch, M., Kaipainen, A., Joukov, V., Meng, X., Lakso, M., Rauvala, H., Swartz, M., Fukumura, D., Jain, R. K., and Alitalo, K. (1997) Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. *Science* **276,** 1423–1425
7. Joukov, V., Sorsa, T., Kumar, V., Jeltsch, M., Claesson-Welsh, L., Cao, Y., Saksela, O., Kalkkinen, N., and Alitalo, K. (1997) Proteolytic processing regulates receptor specificity and activity of VEGF-C. *EMBO J.* **16,** 3898–3911
8. Stacker, S. A., Stenvers, K., Caesar, C., Vitali, A., Domagala, T., Nice, E., Roufail, S., Simpson, R. J., Moritz, R., Karpanen, T., Alitalo, K., and Achen, M. G. (1999) Biosynthesis of vascular endothelial growth factor-D involves proteolytic processing which generates non-covalent homodimers. *J. Biol. Chem.* **274,** 32127–32136
9. Tamagnone, L., Artigiani, S., Chen, H., He, Z., Ming, G. I., Song, H., Chedotal, A., Winberg, M. L., Goodman, C. S., Poo, M., Tessier-Lavigne, M., and Comoglio, P. M. (1999) Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. *Cell* **99,** 71–80
10. Moyon, D., Pardanaud, L., Yuan, L., Breant, C., and Eichmann, A. (2001) Plasticity of endothelial cells during arterial-venous differentiation in the avian embryo. *Development* **128,** 3359–3370
11. Herzog, Y., Kalcheim, C., Kahane, N., Reshef, R., and Neufeld, G. (2001) Differential expression of neuropilin-1 and neuropilin-2 in arteries and veins. *Mech. Dev.* **109,** 115–119
12. Yuan, L., Moyon, D., Pardanaud, L., Breant, C., Karkkainen, M. J., Alitalo, K., and Eichmann, A. (2002) Abnormal lymphatic vessel development in neuropilin 2 mutant mice. *Development* **129,** 4797–4806
13. Soker, S., Takashima, S., Miao, H. Q., Neufeld, G., and Klagsbrun, M. (1998) Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell* **92,** 735–745
14. Mäkinen, T., Olofsson, B., Karpanen, T., Hellman, U., Soker, S., Klagsbrun, M., Eriksson, U., and Alitalo, K. (1999) Differential binding of vascular endothelial growth factor B splice and proteolytic isoforms to neuropilin-1. *J. Biol. Chem.* **274,** 21217–21222
15. Ober, E. A., Olofsson, B., Makinen, T., Jin, S. W., Shoji, W., Koh, G. Y., Alitalo, K., and Stainier, D. Y. (2004) Vegfc is required for vascular development and endoderm morphogenesis in zebrafish. *EMBO Rep.* **5,** 78–84
16. Migdal, M., Huppertz, B., Tessler, S., Comforti, A., Shibuya, M., Reich, R., Baumann, H., and Neufeld, G. (1998) Neuropilin-1 is a placenta growth factor-2 receptor. *J. Biol. Chem.* **273,** 22272–22278
17. Gluzman-Poltorak, Z., Cohen, T., Herzog, Y., and Neufeld, G. (2000) Neuropilin-2 and neuropilin-1 are receptors for the 165-amino acid form of vascular endothelial growth factor (VEGF) and of placenta growth factor-2, but only neuropilin-2 functions as a receptor for the 145-amino acid form of VEGF. *J. Biol. Chem.* **275,** 18040–18045
18. Karkkainen, M. J., Saaristo, A., Jussila, L., Karila, K. A., Lawrence, E. C., Pajusola, K., Bueler, H., Eichmann, A., Kauppinen, R., Kettunen, M. I., Ylä-Herttuala, S., Finegold, D. N., Ferrell, R. E., and Alitalo, K. (2001) A model for gene therapy of human hereditary lymphedema. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 12677–12682
19. Soker, S., Miao, H. Q., Nomi, M., Takashima, S., and Klagsbrun, M. (2002) VEGF165 mediates formation of complexes containing VEGFR-2 and neuropilin-1 that enhance VEGF165-receptor binding. *J. Cell. Biochem.* **85,** 357–368
20. Kitsukawa, T., Shimono, A., Kawakami, A., Kondoh, H., and Fujisawa, H. (1995) Overexpression of a membrane protein, neuropilin, in chimeric mice causes anomalies in the cardiovascular system, nervous system and limbs. *Development* **121,** 4309–4318
21. Kitsukawa, T., Shimizu, M., Sanbo, M., Hirata, T., Taniguchi, M., Bekku, Y., Yagi, T., and Fujisawa, H. (1997) Neuropilin-semaphorin III/D-mediated chemorepulsive signals play a crucial role in peripheral nerve projection in mice. *Neuron* **19,** 995–1005
22. Kawasaki, T., Kitsukawa, T., Bekku, Y., Matsuda, Y., Sanbo, M., Yagi, T., and Fujisawa, H. (1999) A requirement for neuropilin-1 in embryonic vessel formation. *Development* **126,** 4895–4902
23. Chen, H., Bagri, A., Zupicich, J. A., Zou, Y., Stoeckli, E., Pleasure, S. J., Lowenstein, D. H., Skarnes, W. C., Chedotal, A., and Tessier-Lavigne, M. (2000) Neuropilin-2 regulates the development of selective cranial and sensory nerves and hippocampal mossy fiber projections. *Neuron* **25,** 43–56
24. Giger, R. J., Cloutier, J. F., Sahay, A., Prinjha, R. K., Levengood, D. V., Moore, S. E., Pickering, S., Simmons, D., Rastan, S., Walsh, F. S., Kolodkin, A. L., Ginty, D. D., and Geppert, M. (2000) Neuropilin-2 is required in vivo for selective axon guidance responses to secreted semaphorins. *Neuron* **25,** 29–41
25. Jussila, L., Valtola, R., Partanen, T. A., Salven, P., Heikkila, P., Matikainen, M. T., Renkonen, R., Kaipainen, A., Detmar, M., Tschachler, E., Alitalo, R., and Alitalo, K. (1998) Lymphatic endothelium and Kaposi's sarcoma spindle cells detected by antibodies against the vascular endothelial growth factor receptor-3. *Cancer Res.* **58,** 1599–1604
26. Zhu, Z., Hattori, K., Zhang, H., Jimenez, X., Ludwig, D. L., Dias, S., Kussie, P., Koo, H., Kim, H. J., Lu, D., Liu, M., Tejada, R., Friedrich, M., Bohlen, P., Witte, L., and Rafii, S. (2003) Inhibition of human leukemia in an animal model with human antibodies directed against vascular endothelial growth factor receptor 2. Correlation between antibody affinity and biological activity. *Leukemia* **17,** 604–611
27. Waltenberger, J., Claesson-Welsh, L., Siegbahn, A., Shibuya, M., and Heldin, C. H. (1994) Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *J. Biol. Chem.* **269,** 26988–26995
28. Pajusola, K., Aprelikova, O., Pelicci, G., Weich, H., Claesson-Welsh, L., and Alitalo, K. (1994) Signalling properties of FLT4, a proteolytically processed receptor tyrosine kinase related to two VEGF receptors. *Oncogene* **9,** 3545–3555
29. Karpanen, T., Egeblad, M., Karkkainen, M. J., Kubo, H., Jackson, D. G., Ylä-Herttuala, S., Jäättelä, M., and Alitalo, K. (2001) Vascular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. *Cancer Res.* **61,** 1786–1790
30. Kessler, O., Shraga-Heled, N., Lange, T., Gutmann-Raviv, N., Sabo, E., Baruch, L., Machluf, M., and Neufeld, G. (2004) Semaphorin-3F is an inhibitor of tumor angiogenesis. *Cancer Res.* **64,** 1008–1015
31. Veikkola, T., Jussila, L., Makinen, T., Karpanen, T., Jeltsch, M., Petrova, T. V., Kubo, H., Thurston, G., McDonald, D. M., Achen, M. G., Stacker, S. A., and Alitalo, K. (2001) Signalling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. *EMBO J.* **20,** 1223–1231
32. Jeltsch, M., Karpanen, T., Strandin, T., Aho, K., Lankinen, H., and Alitalo, K. (2006) Vascular endothelial growth factor (VEGF)/VEGF-C mosaic molecules reveal specificity determinants and feature novel receptor binding patterns. *J. Biol. Chem.* **281,** 12187–12195
33. Korpelainen, E. I., Karkkainen, M., Gunji, Y., Vikkula, M., and Alitalo, K. (1999) Endothelial receptor tyrosine kinases activate the STAT signaling pathway: mutant Tie-2 causing venous malformations signals a distinct STAT activation response. *Oncogene* **18,** 1–8
34. Uutela, M., Wirzenius, M., Paavonen, K., Rajantie, I., He, Y., Karpanen, T., Lohela, M., Wiig, H., Salven, P., Pajusola, K.,

Eriksson, U., and Alitalo, K. (2004) PDGF-D induces macrophage recruitment, increased interstitial pressure, and blood vessel maturation during angiogenesis. *Blood* **104,** 3198–3204
35. Makinen, T., Veikkola, T., Mustjoki, S., Karpanen, T., Catimel, B., Nice, E. C., Wise, L., Mercer, A., Kowalski, H., Kerjaschki, D., Stacker, S. A., Achen, M. G., and Alitalo, K. (2001) Isolated lymphatic endothelial cells transduce growth, survival and migratory signals via the VEGF-C/D receptor VEGFR-3. *EMBO J.* **20,** 4762–4773
36. Mamluk, R., Gechtman, Z., Kutcher, M. E., Gasiunas, N., Gallagher, J., and Klagsbrun, M. (2002) Neuropilin-1 binds vascular endothelial growth factor 165, placenta growth factor-2, and heparin via its b1b2 domain. *J. Biol. Chem.* **277,** 24818–24825
37. Giger, R. J., Urquhart, E. R., Gillespie, S. K., Levengood, D. V., Ginty, D. D., and Kolodkin, A. L. (1998) Neuropilin-2 is a receptor for semaphorin IV: insight into the structural basis of receptor function and specificity. *Neuron* **21,** 1079–1092
38. Kolodkin, A. L., Levengood, D. V., Rowe, E. G., Tai, Y. T., Giger, R. J., and Ginty, D. D. (1997) Neuropilin is a semaphorin III receptor. *Cell* **90,** 753–762
39. He, Z., and Tessier-Lavigne, M. (1997) Neuropilin is a receptor for the axonal chemorepellent Semaphorin III. *Cell* **90,** 739–751
40. Le Roy, C., and Wrana, J. L. (2005) Clathrin- and non-clathrin-mediated endocytic regulation of cell signalling. *Nat. Rev. Mol. Cell. Biol.* **6,** 112–126
41. Jekely, G., Sung, H. H., Luque, C. M., and Rorth, P. (2005) Regulators of endocytosis maintain localized receptor tyrosine kinase signaling in guided migration. *Dev. Cell* **9,** 197–207
42. Wang, L., Zeng, H., Wang, P., Soker, S., and Mukhopadhyay, D. (2003) Neuropilin-1-mediated vascular permeability factor/vascular endothelial growth factor-dependent endothelial cell migration. *J. Biol. Chem.* **278,** 48848–48860
43. Serini, G., Valdembri, D., Zanivan, S., Morterra, G., Burkhardt, C., Caccavari, F., Zammataro, L., Primo, L., Tamagnone, L., Logan, M., Tessier-Lavigne, M., Taniguchi, M., Puschel, A. W., and Bussolino, F. (2003) Class 3 semaphorins control vascular morphogenesis by inhibiting integrin function. *Nature* **424,** 391–397
44. Feiner, L., Webber, A. L., Brown, C. B., Lu, M. M., Jia, L., Feinstein, P., Mombaerts, P., Epstein, J. A., and Raper, J. A. (2001) Targeted disruption of semaphorin 3C leads to persistent truncus arteriosus and aortic arch interruption. *Development* **128,** 3061–3070
45. Baldwin, M. E., Catimel, B., Nice, E. C., Roufail, S., Hall, N. E., Stenvers, K. L., Karkkainen, M. J., Alitalo, K., Stacker, S. A., and Achen, M. G. (2001) The specificity of receptor binding by vascular endothelial growth factor-D is different in mouse and man. *J. Biol. Chem.* **276,** 19166–19171
46. Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P., and Ornitz, D. M. (1991) Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell* **64,** 841–848
47. Lyon, M., Deakin, J. A., and Gallagher, J. T. (2002) The mode of action of heparan and dermatan sulfates in the regulation of hepatocyte growth factor/scatter factor. *J. Biol. Chem.* **277,** 1040–1046
48. Soker, S., Fidder, H., Neufeld, G., and Klagsbrun, M. (1996) Characterization of novel vascular endothelial growth factor (VEGF) receptors on tumor cells that bind VEGF165 via its exon 7-encoded domain. *J. Biol. Chem.* **271,** 5761–5767
49. Fuh, G., Garcia, K. C., and de Vos, A. M. (2000) The interaction of neuropilin-1 with vascular endothelial growth factor and its receptor flt-1. *J. Biol. Chem.* **275,** 26690–26695
50. Robinson, C. J., and Stringer, S. E. (2001) The splice variants of vascular endothelial growth factor (VEGF) and their receptors. *J. Cell Sci.* **114,** 853–865
51. Dougher, M., and Terman, B. I. (1999) Autophosphorylation of KDR in the kinase domain is required for maximal VEGF-stimulated kinase activity and receptor internalization. *Oncogene* **18,** 1619–1627
52. Shen, J., Samul, R., Zimmer, J., Liu, H., Liang, X., Hackett, S., and Campochiaro, P. A. (2004) Deficiency of neuropilin 2 suppresses VEGF-induced retinal neovascularization. *Mol. Med.* **10,** 12–18
53. Di Guglielmo, G. M., Le Roy, C., Goodfellow, A. F., and Wrana, J. L. (2003) Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover. *Nat. Cell Biol.* **5,** 410–421

Received for publication November 1, 2005.
Accepted for publication March 14, 2006.