Neuropilin-1-mediated Vascular Permeability Factor/Vascular Endothelial Growth Factor-dependent Endothelial Cell Migration*

Received for publication, September 10, 2003
Published, JBC Papers in Press, September 26, 2003, DOI 10.1074/jbc.M310047200

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Neuropilin-1 (NRP-1) has been found to be expressed by endothelial cells and tumor cells as an isoform-specific receptor for vascular permeability factor/vascular endothelial growth factor (VEGF). Previous studies were mainly focused on the extracellular domain of NRP-1 that can bind to VEGF165 and, thus, enables NRP-1 to act as a co-receptor for VEGF165, which enhances its binding to VEGF receptor-2 and its bioactivity. However, the exact functional roles and related signaling mechanisms of NRP-1 in angiogenesis are not well understood. In this study we constructed a chimeric receptor, EGNP-1, by fusing the extracellular domain of epidermal growth factor receptor to the transmembrane and intracellular domains of NRP-1 and transduced it into HUVECs with a retroviral expression vector. We observed that NRP-1/EGNP-1 mediates ligand-stimulated migration of human umbilical vein endothelial cells (HUVECs) but not proliferation. Our results show that NRP-1 alone can mediate HUVEC migration through its intracellular domain, and its C-terminal three amino acids (SEA-COOH) are essential for the process. We demonstrate that phosphatidylinositol 3-kinase inhibitor Ly294002 and the p85 dominant negative mutant can block NRP-1-mediated HUVEC migration. NRP-1-mediated migration can be significantly reduced by overexpression of the dominant negative mutant of RhoA (RhoA-18N). In addition, Gαq family proteins and Gβγ subunits are also required for NRP-1-mediated HUVEC migration. These results show for the first time that NRP-1 can independently promote cell signaling in endothelial cells and also demonstrate the importance of last three amino acids of NRP-1 for its function.

Angiogenesis, the formation of vascular networks by endothelial cells (ECs) sprouting from the vascular bed, occurs in many physiological or pathological processes (1). Vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) plays a major role in the regulation of angiogenesis; it is regarded as a key contributor to the growth of cancer and vascular disease (2). VPF/VEGF activities are mediated by high affinity receptor tyrosine kinases that are associated primarily with ECs (2). Two important VPF/VEGF binding receptor tyrosine kinases, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flik-1), have been identified, both of which are functionally active during angiogenesis. Recent studies have found a third VPF/VEGF receptor, neuropilin-1 (NRP-1), that is expressed by ECs and tumor cells (3, 4).

NRP-1 is a 130–135-kDa cell surface glycoprotein. It was originally characterized as a semaphorin III receptor that is important for guiding neural development (5, 6). There is also evidence that NRP-1 mediates angiogenesis. NRP-1-null mice were found to be embryonic lethal and exhibit cardiovascular defects (7). Furthermore, overexpression of NRP-1 in mice resulted in excessive capillary and blood vessel formation and hemorrhaging in embryos (8). NRP-1 also contributes to tumor angiogenesis. Induction of NRP-1 expression in tumor cells in vivo resulted in larger and more vascular tumors (9). The stronger evidence for the role of NRP-1 in angiogenesis is its expression on ECs in the adult uterus (10) and on new vasculature in healing wounds (11).

Previously, it has been demonstrated that expression of NRP-1 on ECs enhanced the VEGF165 binding to VEGFR-2 and VEGFR-2-mediated chemotactic activity of VEGF165 (4). This suggests that NRP-1 is a co-receptor for VPF/VEGF, which has led to postulate that NRP-1 is involved in VPF/VEGF-mediated angiogenesis in vivo. Further studies on NRP-1 function have also provided evidence for its role in EC as a critical VEGFR-2 co-receptor that facilitates VPF/VEGF-mediated signaling through this tyrosine kinase-linked receptor (12). Additionally, the study in Dunning rat prostate carcinoma AT2.1 cells which express NRP-1 but not VEGFR-2 found that the expression of NRP-1 resulted in enlarged tumors associated with substantially enhanced tumor angiogenesis (9). A recent study showed that NRP-1 bound with high affinity to VEGF165 and that this interaction inhibited the binding of VEGF165 to NRP-1 (13). Interestingly, a study by Bachelder et al. (14) first demonstrated that NRP-1 supported VPF/VEGF autocrine function in cells lacking VEGF-2 expression. All this evidence raises the possibility that NRP-1 functions either alone or in concert with the extracellular domain of the EGF receptor and the transmembrane/intracellular domains of VEGFR-1; EGNP-1, the mutant of EGNP-1 by deleting the C-terminal three amino acids of NRP-1 (SEA-COOH); Pi3K, phosphatidylinositol 3-kinase; FACS, fluorescence-activated cell sorting; NIP, NRP-1-interacting protein; EBM, EC basic medium; PBS, phosphate-buffered saline; GST, glutathione S-transferase; TRBD, GST-Rhotekin Rho binding domain; DN, dominant negative.

*This work was supported by National Institutes of Health Grants HL70657, HL072178, and CA78383, the American Cancer Society, Department of Defense Breast Cancer Program (to D. M.), and by National Institutes of Health Grant CA 45548 (to S. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡The abbreviations used are: EC, endothelial cell; VPF, vascular permeability factor; VEGF, vascular endothelial growth factor; EGF, epidermal growth factor; EGF, EGF receptor HUVEC, human umbilical vein endothelial cell; NRP-1, neuropilin-1; EGNP-1, the fusion protein with the extracellular domain of the EGF receptor and the transmembrane/intracellular domains of NRP-1; EGDR, the fusion protein with the extracellular domain of the EGF receptor and the transmembrane/intracellular domain of VEGFR-2; EGLT, the fusion protein with the extracellular domain of the EGF receptor and the transmembrane/intracellular domain of VEGFR-2; EGNP-1, the fusion protein with the extracellular domain of the EGF receptor and the transmembrane/intracellular domain of VEGFR-2; EGNP-1, the fusion protein with the extracellular domain of the EGF receptor and the transmembrane/intracellular domain of VEGFR-2; EGNP-1, the fusion protein with the extracellular domain of the EGF receptor and the transmembrane/intracellular domain of VEGFR-2; EGNP-1, the fusion protein with the extracellular domain of the EGF receptor and the transmembrane/intracellular domain of VEGFR-2; EGNP-1, the fusion protein with the extracellular domain of the EGF receptor and the transmembrane/intracellular domain of VEGFR-2.
other tyrosine kinase-linked receptors to transduce the VPF/VEGF signaling in different cells. However, neither of the above signaling pathways has been investigated to date.

NRP-1 contains a relatively large extracellular domain of 860 amino acids, a very short transmembrane domain of 23 amino acids, and an intracellular domain of 40 amino acids. Previous studies were mainly focused on the extracellular domain that consists of five domains: a1/a2, b1/b2, and c (a MAM domain). The b1/b2 domains of NRP-1 contain the epitopes for both VPF/VEGF 165 and the positively charged C terminus of semaphorins (15–17) that are the structural basis for NRP-1 function as a VEGFR-2 co-receptor for VPF/VEGF signaling. Research from the nervous system showed that the intracellular domain of NRP-1 was not required for semaphorin signaling (16), and it did not contain sequences predictive of enzymatic activity nor sequences predicted to be involved in coupling to intracellular signaling molecules. Nevertheless, the transmembrane and intracellular domains of NRP-1 share >90% amino acid identity across species (18, 19), which suggests an important role for this domain in terms of the NRP-1 functions.

Moreover, the C-terminal three amino acids of NRP-1 (SEA) to human, were conserved from Xenopus to human, were responsible for binding to a PS-D-5-Dlg/ZO (PDZ) domain (20, 21).

Because VEGFR-1, VEGFR-2, and NRP-1 are all expressed on ECs, it is difficult to delineate the distinct biological functions and signaling pathways of each induced in ECs by VPF/VEGF. Therefore, to elucidate the respective roles of these receptors in ECs, an approach engineering the chimeric construct of each of the receptors by replacing the extracellular domain of each with the extracellular domain of epidermal growth factor receptor (EGFR), was established previously in our laboratory (22). In this study, using a chimeric receptor EGNP-1 (fusing the extracellular domain of the EGFR and the transmembrane/intracellular domains of NRP-1) we examined the distinct biological function of the intracellular domain of NRP-1 and its signaling pathways in vascular endothelium. We found that NRP-1 alone can mediate HUVEC migration but not proliferation, and the C-terminal three amino acids of NRP-1 (SEA-COOH) are required for NRP-1-mediated HUVEC migration. The activation of RhoA can be mediated by NRP-1 through Gs, family proteins, Gβγ subunits, and PI3K pathways that are required for HUVEC migration.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human EGF, human VEGF, primary HUVECs, EGM-MV bullet kit and EC basic medium (EBM) were purchased from Clonetics (San Diego, CA). Mouse monoclonal antibody against the EGFR N-terminal domain, goat polyclonal antibody against the NRP-1 C-terminal domain, and rabbit polyclonal antibody against RhoA were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). [3H]Thymidine was purchased from PerkinElmer Life Sciences. Tris-HCl, pH 7.4, 0.15 M NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, which contains 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 0.5% aprotinin, and 2 μg/ml pepstatin A. 500 μg of lysate protein was incubated with 1 μg of antibody at 4 °C for 2 h and then with 50 μl of protein G-conjugated agarose beads at 4 °C for 2–5 h. After washing the beads with the same buffer, immunoprecipitates were resuspended in 2× SDS sample buffer for Western blot analysis. All experiments were repeated at least three times.

**FACS Analysis**—For detecting detached, serum-starved HUVECs were incubated with 4 μl of collagenase solution (0.2 mg/ml collagenase, 0.2 mg/ml soybean trypsin inhibitor, 1 mg/ml bovine serum albumin, and 2 mm EDTA in PBS) at 37 °C for 30 min. Cell pellets were washed with cold PBS containing 0.1% bovine serum albumin, and 5 × 10⁶ cells were suspended in 40 μl of the same buffer containing 4 μg of mouse anti-EGFR-N antibody or mouse IgG and incubated on ice for 1 h. The cells were washed again and resuspended in 40 μl of the same buffer with 2.5 μg/ml fluorescein isothiocyanate-conjugated anti-mouse IgG antibody. After incubation at 4 °C for 30 min, cells were washed and resuspended in 400 μl of the same buffer. FACScan analysis was carried out in a Calibur instrument (BD Biosciences) with Cellquest software.

**Migration Assay**—Serum-starved HUVECs (with or without retrovirus infection) were stained with calcein-AM (25 μg of calcein-AM was dissolved in 5 μl of MeSO and then added into 4 ml of EBM containing 0.1% bovine serum albumin/100-mm plate) at 37 °C for 30 min. The cells were then detached from culture plates and analyzed in FACScan analysis. Then cells were seeded as 1 × 10⁶/well in 500 μl of EBM with 0.1% fetal bovine serum into the transwells coated with Vitrogen (30 μg/ml), and the transwells were inserted into a 24-well plate containing 750 μl of the same medium. The cells were incubated at 37 °C for 45 min to allow them to attach. Afterward, VPF/VEGF or EGF was added at a final concentration of 10 ng/ml, and an additional 2-h incubation was performed. The migrated, stained cells were counted in a spectrofluorometer (Spectrafluor; TECAN) with Delta Soft 3 software. The standard curve was made under the same conditions with cells over a range of 3 × 10⁵ to 5 × 10⁶ cells/well. For migration inhibition experiments, various inhibitors were added at different times before Coating. Both EGF or EGF stimulated, but not unstimulated, migration was inhibited by the mean ± S.D. of quadruplicate values. All experiments were repeated at least three times.

**Proliferation Assays**—A proliferation assay was carried out as described previously (22). 2 × 10³ cells were seeded into each well in a
24-well plate. After 24 h, the cells were infected with 200 μl of retrovirus in 800 μl of fresh medium as described above. After 2 days, the cells were serum-starved for 24 h and then stimulated with 10 ng/ml VPF/VEGF or 10 ng/ml EGF for 20 h. The assay was carried out as previously described (22). 1 μCi/ml [3H]thymidine was added to each well. After 4 h of incubation at 37°C, cells were washed with cold PBS, fixed with 100% cold methanol at 4°C for 15 min, precipitated with 10% cold trichloracetic acid at 4°C for 15 min, washed with double distilled H2O 3 times, and lysed with 200 μl of 0.1 N NaOH at room temperature for 30 min. [3H]Thymidine incorporation was measured in scintillation solution. Data are expressed as the mean ± S.D. of quadruplicate values. All experiments were repeated at least three times.

RhoA Activation Assay—RhoA activation assay was carried out as described previously (22). The glutathione-S-transferase (GST)-Rho-tektn Rho binding domain (TRBD) fusion protein was kindly provided by Dr. Martin Schwartz (Scripps Institute). In brief, pGST-TRBD bacteria were grown and induced with isopropylthiogalactoside. The bacterial suspensions were aliquoted per 50 ml and then harvested and frozen at –80°C. To prepare the GST-TRBD beads, each aliquot of frozen bacteria was resuspended in 2 ml of cold PBS, and then 20 μl of 1× dithiothreitol, 20 μl of 0.2× phenylmethylsulfonyl fluoride, and 40 μl of 50 mg/ml lysozyme were added and incubated on ice for 30 min. Afterward, 225 μl of 10% Triton X-100, 22.5 μl of 1× MgCl2, and 22.5 μl of 20000 units/ml DNase were added, and the sample was incubated on ice for another 30 min. The supernatant was collected and incubated with 100 μl of glutathione-coupled Sepharose 4B beads (Amersham Biosciences) at 4°C for 45 min. The beads were then washed with bead washing buffer (PBS with 10 mM dithiothreitol and 1% Triton X-100) and resuspended in the same buffer to emit 50% bead slur.

Meanwhile, 24 h serum-starved HUVECs with retroviral infection were stimulated with 10 ng/ml EGF at different times. Stimulation was stopped by adding cold PBS. Then the cells were lysed with lysis buffer (150 mM NaCl, 0.8 mM MgCl2, 5 mM EGTA, 1% Igepal, 50 mM HEPES, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin, and 10 μg/ml aprotinin). The supernatant was isolated and incubated with 50 μl of GST-TRBD beads at 4°C for 45 min. Protein bound to beads was washed with AP wash buffer (50 mM Tris-HCl, pH 7.2; 1% Triton X-100, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) and analyzed by SDS-PAGE with antibody against RhoA. For inhibition experiments, inhibitors were added as indicated. All experiments were repeated at least three times.

RESULTS

Construction, Transduction, and Recombinant Expression of EGNP-1 Chimeric Receptor—Previously, our laboratory developed EGLT and EGDR fusions with the extracellular domain of EGFR to the transmembrane/intracellular domains of VEGFR-1 and VEGFR-2, respectively, and expressed them in HUVECs with a retrovirus expression vector (22–24). At 80% confluence, HUVECs do not express EGFR, and therefore, the fusion receptor can be used to distinguish the signaling pathway that is mediated by VEGFR-1 and VEGFR-2. In this study, to investigate the biological activities and the specific signaling events mediated by NRP-1 in ECs, we developed a chimeric receptor, EGNP-1, by fusing the extracellular domain of EGFR to the transmembrane and intracellular domains of NRP-1 (Fig. 1a). Using a retroviral expression vector pMMP, the chimeric receptor EGNP-1 was transduced into HUVECs. Cells transduced with LacZ were used as control. Western blot (IB) analysis was performed on these cell lysates with an antibody to the C terminus of NRP-1 (anti-NRP-1-C). The results showed the specific bands of endogenous NRP-1 in both cells, but the EGNP-1 band only in EGNP-1 transduced HUVECs (Fig. 1b, the left two lanes). Immunoprecipitation (IP) with an antibody against the EGFR N terminus (anti-EGFR-N) and then Western blot with anti-NRP-1-C were also carried out and further demonstrated that the clear specific band of EGNP-1 that expressed only in EGNP-1 transduced HUVECs (Fig. 1b, the two right lanes). Of importance, the expression of endogenous NRP-1 and recombinant EGNP-1 expression are almost at similar levels. To determine the expression of EGNP-1 on HUVEC surfaces, FACS analysis was performed using a mouse monoclonal antibody specific for the N terminus of EGFR and normal mouse IgG as control. HUVECs were transduced with LacZ (negative control), EGDR (positive control), and EGNP-1. The results showed that about 90% of transduced cells expressed EGDR and more than 70% of transduced cells expressed EGNP-1 (Fig. 1c). EGFR expression was not detected in LacZ-transduced HUVECs as previously shown (22).

EGNP-1 Mediates HUVEC Migration but Not Proliferation—First, we examined whether EGNP-1 could mediate the migration and proliferation of HUVECs. Migration assays were carried out on HUVECs transduced with EGNP-1, EGDR, EGLT, and LacZ. As shown in Fig. 2a, I, EGF induced the migration to an almost equivalent level in HUVECs that had been transduced with either EGNP-1 or EGDR but not to that of native HUVECs and HUVECs transduced with EGLT or LacZ. All of the cells showed a similar response to VPF/VEGF stimulation, indicating that the cells had good responsiveness. The results from Western blot analysis demonstrated all the chimeric receptors were expressed at relatively comparable levels in HUVECs (Fig. 2a, III). These results indicated that EGNP-1 can mediate the ligand-dependent migration of HUVECs. Furthermore, proliferation assays were also performed on these HUVECs as above. As shown in Fig. 2a, II, VPF/VEGF treatment stimulated more than 3-fold in parental HUVECs, and EGF treatment promoted 4-fold in EGLT-transduced HUVECs in trichloracetic acid-precipitable [3H]thymidine incorporation. On the other hand, there was no response in EGF-stimulated EGNP-1- or LacZ-transduced HUVECs or parental HUVECs. The results suggest that EGNP-1 is not required for ligand-dependent HUVEC proliferation.

To quantitatively characterize the medication function of EGNP-1 in HUVEC migration, we infected HUVECs by varying the number of retrovirus particles containing EGNP-1. Fig. 2b, II, illustrated that the transduced cells expressed progressively increased levels of EGNP-1. Stimulating the transduced cells with EGF indicated that there was a positive correlation between its migration and levels of EGNP-1 (Fig. 2b, I and II). This quantitative result further demonstrated that NRP-1/EGNP-1 mediate EGF-induced HUVEC migration.

To examine whether EGNP-1 alone can mediate migration of HUVECs, EGDR mutants EGDR (Y951F) and EGDR (Y1059F) (site-directed mutagenesis in tyrosine residue 951 and 1059 of EGLT essential for VPF/VEGF induced HUVEC migration and proliferation, respectively) and EGLT mutants EGLT (793stop) (a stop codon at tyrosine residue 794 of EGLT essentially for VEGFR-1 kinase domain), were respectively, transduced into HUVECs with and without EGNP-1. We found that the migration of HUVECs co-transduced with EGNP-1 and EGDR mutants or EGNP-1 and EGLT mutants showed no difference from that of cells transduced with EGNP-1 alone (Fig. 3a, I). For all cases, the expression of EGNP-1 was at similar levels (Fig. 3a, II). To confirm these results, we inhibited the VEGFR-2 function by utilizing anti-VEGFR-2 antibody on parental or EGNP-1-transduced HUVECs. The results indicated that VEGFR-2 antibody inhibited native HUVEC migration induced by VPF/VEGF but not EGNP-1-transduced HUVEC migration induced by EGF (Fig. 3b). Taken together these data demonstrate that the function of NRP-1/EGNP-1-mediated HUVEC migration can be VEGFR-2- or VEGFR-1-independent.

Signaling Downstream of NRP-1-mediated Endothelial Cell Migration—To examine the role of PI3K-signaling pathways in NRP-1-mediated HUVEC migration, Ly294002, a PI3K inhibitor, was used. After treatment of EGNP-1-transduced
Fig. 1. Construction and expression of the chimeric receptor EGNP-1 in HUVECs. a, the extracellular domain of the EGFR was fused to the transmembrane and intracellular domains of NRP-1 and NRP-1ΔSEA to create chimeric fusion receptors EGNP-1 and EGNP-1ΔSEA, respectively. b, the extracts of HUVECs that had been transduced with LacZ and EGNP-1, respectively, were immunoblotted (IB) with an antibody against the C terminus of NRP-1 (Anti-NRP-1-C) (left two lanes) or immunoprecipitated (IP) with an antibody against the N terminus of EGFR (Anti-EGFR-N) and immunoblotted as above (right two lanes). c, FACS analysis of HUVECs transduced with LacZ, EGDR, and EGNP-1 with fluorescent anti-EGFR-N or mouse IgG. FL1-H, fluorescence intensity. FL1-H represents the expression level of receptor.
HUVECs with Ly294002, a significant decrease of HUVEC migration in response to EGF was observed (Fig. 4a). PI3K contains a kinase subunit (p110) and an inhibitory subunit (p85) and functions in tyrosine kinase receptor signaling pathways (25). Ligand-activated receptors interact with the p85 subunit, releasing the p110 subunit in an active form. We used the dominant negative mutants of the p85 subunit (p85(DN)) and the constitutive activated mutant of PI3K (p110CAAX) to determine whether PI3K is required for EGNP-1-mediated migration. The results showed that the co-transduced HUVECs expressed similar levels of EGNP-1 (Fig. 4b, II), but the co-transduction of HUVECs with EGNP-1 and p85(DN) reduced EGF-stimulated migration as compared with that of HUVECs co-transduced with EGNP-1 and LacZ (Fig. 4b, I). However, the p110CAAX increased EGNP-1-mediated migration induced by EGF. These results indicated that EGNP-1-mediated ligand-dependent migration of HUVECs is through the PI3K signaling pathway.

Previous work on the nervous system has shown that NRP-1 may be involved in the regulation of vesicular trafficking by association with the G protein-coupled signaling pathways (20). Hence, we analyzed whether G proteins are involved in the signaling of NRP-1/EGNP-1-mediated HUVEC migration. It has been shown that pertussis toxin inhibits VEGF-mediated microphage migration and HUVEC anti-proliferation stimulated by VPF/VEGF (26, 27), which suggest that pertussis toxin-sensitive G proteins may participate in VPF/VEGF-dependent signaling. Recent studies from our laboratory have shown that Gq family proteins are important for VPF/VEGF-stimulated HUVEC proliferation and migration. "Serum-starved EGNP-1-transduced HUVECs were pretreated with 100 ng/ml pertussis toxin for 16 h or 1 μM TATFGp (a cell-permeable fusion peptide TAT-FLAG-Gp that consists of a TAT peptide, an epitope FLAG sequence, and Gp antagonist 2A; Gp antagonist 2A can specifically inhibit function of Gq family proteins in vitro (29, 30)" for 5 min and then stimulated with EGF. The data show that pretreatment with pertussis toxin did not have any effect on EGNP-1-mediated HUVEC migration, but TATFGp significantly decreased the migration (Fig. 5a).

These results suggest that Gq family proteins are involved in EGNP-1-mediated HUVEC migration. It is known that after activation heterotrimeric G proteins dissociate into α and βγ subunits that can trigger several downstream signaling pathways (31, 32). Gβγ is also known to be involved in VEGFR-1- and VEGFR-2-mediated signaling pathways (23, 27). Therefore, we further tested the role of free Gβγ subunits in NRP-1/EGNP-1 mediated HUVEC migration. hβARK1 (495) corresponds to the C-terminal domain of human β ARK1 that physically interacts with free Gβγ and, therefore, acts as a ...
Fig. 3. NRP-1/EGNP-1 alone mediates HUVEC migration. a, HUVECs transduced with EGNP-1 or co-transduced with EGNP-1 and EGDR (Y951F), EGDR (Y1059F), EGLT (793stop), or LacZ were stimulated with 10 ng/ml EGF for migration assays (I). The expression of EGNP-1 was checked by immunoblotting (IB) with anti-NRP-1-C (II). b, HUVECs and HUVECs transduced with EGNP-1 were treated with different concentrations of anti-VEGFR-2 (0, 1, 5 μg/ml), then stimulated with 10 ng/ml VPF/VEGF or 10 ng/ml EGF for migration assays.
specific intracellular Gβγ antagonist by inhibiting Gβγ-mediated downstream events (33, 34). Our results showed that overexpression of hβARK1 (495) almost completely reduced the migration of HUVECs mediated by EGNP-1 (Fig. 5b, I), although approximately the same levels of EGNP-1 were expressed in all the co-transduced cells (Fig. 5b, II), indicating that Gβγ subunits are required for EGNP-1-mediated HUVEC migration.

The RhoA family of the small GTPase superfamily has been shown to play an important role in cell growth and migration (35).
FIG. 5. Involvement of G protein and Gβγ subunits in NRP-1/EGNP-1-mediated HUVEC migration. a, migration assay. HUVECs transduced with EGNP-1 were pretreated with and without pertussis toxin (100 ng/ml) for 16 h or TATGFp (1 μM) for 5 min, then stimulated with 10 ng/ml EGF. b, HUVECs were transduced with EGNP-1 or co-transduced with EGNP-1 and hβARK1 (495) or LacZ. With these cells the migration assay was carried out with 10 ng/ml EGF stimulation (I), and the immunoblotting (IB) was carried out with anti-NRP-1-C (II).
Recently, we reported that CDC42 and Rac1 are required for VEGFR-1-mediated HUVEC anti-proliferation (27), whereas RhoA and Rac1 are important for VEGFR-2-mediated HUVEC migration (23). Using the fragments encoding the genes of the dominant negative mutants of CDC42 (CDC42-17N), Rac1 (Rac1-17N), and RhoA (RhoA-19N) that were previously subcloned to a retroviral vector pMMP in our laboratory (27), we examined whether Rho family proteins were involved in NRP-1-mediated HUVEC migration. HUVECs were co-transduced with EGNP-1 and CDC42-17N, Rac1-17N, or RhoA-19N. Fig. 6 shows all the co-transduced cells expressed EGNP-1 at relatively comparable levels. Fig. 6I shows that compared with LacZ-transduced cells, the CDC42-17N- or Rac1-17N-transduced cells did not influence EGNP-1-mediated HUVEC migration, but the RhoA-19N-transduced HUVECs showed significant inhibition of ligand-induced migration. Taken together, our results suggest that RhoA is involved in ligand-dependent migration of HUVECs mediated by EGNP-1.

**NRP-1/EGNP-1 and Related Intracellular Signaling Molecules Mediate Activation of RhoA**—Because the dominant negative mutant of RhoA (RhoA-19N) decreases EGF-induced HUVEC migration mediated by EGNP-1, we further examined whether EGNP-1 can mediate ligand-dependent RhoA activation. The activity of RhoA was measured by a pull-down assay using a GST-TRBD fusion protein that binds only to the GTP-bound form of RhoA. Serum-starved HUVECs transduced with EGNP-1 or LacZ were stimulated with 10 ng/ml EGF for different intervals as indicated. Cellular extracts were incubated with freshly prepared GST-TRBD beads. Proteins bound to the beads were subjected to Western blot analysis using the antibodies against RhoA. The results show that RhoA was not activated by EGF in LacZ transduced HUVECs but was activated by EGF in EGNP-1-transduced HUVECs as early as 0.5 min and remained high at 5 min after EGF stimulation (Fig. 7a).
To test the signaling molecules that mediate RhoA activation, EGNP-1-transduced HUVECs were pretreated with a PI3K inhibitor, Ly294002, for 5 min and then stimulated with 10 ng/ml EGF for 1 min. Cellular extracts were used to measure the activation of RhoA. The data show that Ly294002 inhibited RhoA activation in EGNP-1-transduced HUVECs after EGF stimulation (Fig. 7b, I). HUVECs transduced with EGNP-1 were incubated with and without PI3K inhibitor Ly294002 (25 μM) for 5 min before the addition of 10 ng/ml EGF for 1 min. II, HUVECs co-transduced with EGNP-1 and LacZ or p85(DN) were stimulated with and without 10 ng/ml EGF for 1 min. The data illustrated that overexpression of hβARK1 (495) inhibited the activation of RhoA (Fig. 7c, II), thus indicating that Gβγ subunits are required for EGNP-1-mediated RhoA activation.

The C-terminal Three Amino Acids of NRP-1 (SEA-COOH) Are Essential for Ligand-induced EGNP-1-mediated HUVEC Migration—To confirm the specific signaling transduction induced by NRP-1 in HUVECs, a chimeric mutant receptor was created by deleting the C-terminal three amino acids of NRP-1 (SEA-COOH) and designated EGNP-1 SEA (Fig. 1a). HUVECs transduced with LacZ, EGNP-1, and EGNP-1 SEA expressed equal amounts of NRP-1 and, accordingly, expressed equal amounts of EGNP-1 and EGNP-1 SEA (Fig. 8a, I). To confirm that the mutant protein is expressed on the cell surface.
as a receptor, the transduced cells were subjected to FACS analysis with anti-EGFR-N and normal mouse IgG as a control. The results indicated that about 70% of HUVECs infected with EGNP-1/H9004 SEA and about 90% of HUVECs infected with EGDR expressed the receptors on the cell surface, whereas no EGFR was detected on HUVECs transduced with LacZ (Fig. 8a Ii). Next we investigated the effect of the mutation on EGNP-1-mediated HUVEC migration. As shown in Fig. 8b Ii, HUVECs transduced with EGNP-1/H9004 SEA showed a similar response to VPF/VEGF stimulation, indicating that the lack of migration response of EGNP-1/H9004 SEA is not due to the defect of the cell. These results indicated that the C-terminal three amino acids of NRP-1 (SEA-COOH) are essential for ligand-induced HUVEC migration.

We further examined whether EGNP-1/H9004 SEA has any effect on RhoA activation in HUVECs. RhoA activity assay was carried out in HUVECs transduced with EGNP-1/H9004 SEA. As shown in Fig. 8c, EGNP-1/H9004 SEA did not mediate RhoA activation in response to EGF stimulation. This suggested that the C-terminal three amino acids of NRP-1 (SEA-COOH) are required for the signaling transduction of EGNP-1-mediated HUVEC migration.

Fig. 8. Effect of the C-terminal three amino acids of NRP-1 (SEA-COOH) on HUVEC migration. a, HUVECs were transduced with LacZ, EGNP-1, and EGNP-1/H9004 SEA. I, the extracts of these cells were immunoblotted (IB) with anti-NRP-1-C (left three lanes) or immunoprecipitated (IP) with anti-EGFR-N and immunoblotted with anti-NRP-1-C (right three lanes). II, FACS analysis of cell surface expression of EGNP-1/H9004 SEA in these cells with fluorescent anti-EGFR-N or mouse IgG. b, HUVECs or HUVECs transduced with LacZ, EGNP-1, and EGNP-1/H9004 SEA were stimulated with 10 ng/ml VPF/VEGF or 10 ng/ml EGF for migration assay (I). Immunoblotting was carried out with anti-NRP-1-C to check the expression levels of the target proteins in these cells (II). c, effect of EGNP-1/H9004 SEA on RhoA activity. HUVECs transduced with EGNP-1/H9004 SEA were stimulated with 10 ng/ml EGF for different lengths of time (0, 0.5, 1, and 5 min).

DISCUSSION

Research on neuropilins and their biological functions is just at the beginning. Although it is known that NRP-1 acts as an important modulator of VPF/VEGF function during vasculogenesis and angiogenesis, its exact functional roles and related signaling mechanisms in these processes are still unclear (4, 9, 12, 14). This study has elucidated some of the functional roles of NRP-1 in EC migration and explored related signaling mechanisms, the implications of which are discussed below.

This study provides direct evidence for the mediation function of NRP-1 in VPF/VEGF-dependent migration in HUVECs. To probe the functional role of NRP-1 in early passaged ECs, we used an approach previously established in our laboratory (22) to develop a chimeric receptor EGNP-1 by fusing the extracellular domain of the EGF receptor with the transmembrane and intracellular domains of NRP-1. However, HUVECs transduced with EGNP-1/H9004 SEA showed a similar response to VPF/VEGF stimulation, indicating that the lack of migration response of EGNP-1/H9004 SEA is not due to the defect of the cell. These results indicated that the C-terminal three amino acids of NRP-1 (SEA-COOH) are essential for ligand-induced HUVEC migration.

Neuropilin-1, a transmembrane receptor that binds neuropilin-1 ligands, has been shown to play a crucial role in the regulation of angiogenesis and vasculogenesis. However, the specific mechanisms by which neuropilin-1 regulates EC migration remain largely unknown.

In this study, we investigated the role of the C-terminal three amino acids of NRP-1 (SEA-COOH) in regulating EC migration. We used a chimeric receptor, EGNP-1, which is composed of the extracellular domain of the EGF receptor and the transmembrane and intracellular domains of NRP-1. EGNP-1-H9004 SEA was shown to mediate EGF-induced HUVEC migration but not proliferation. Furthermore, we identified that EGF-induced HUVEC migration is dose-dependent. This phenomenon, although observed in different experimental approaches and different types of endothelium, agrees with and extends a recent study by Matthis et al. (11), where it was described that the migration of human microvascular endothelial cells to VEGF165 was severely inhibited in vitro in the presence of anti-NRP-1 antibody.
and this inhibition was dose-dependent. These converging findings consistently support that NRP-1 mediates VPF/VEGF-induced EC migration.

This study examined whether NRP-1 alone can mediate EC migration. There are three lines of evidence in our results that support this functional role of NRP-1. First, we found that EGF stimulates a nearly equivalent extent of increase of migration in HUVECs transduced with EGNP-1 or EGDR (another chimeric receptor identified to represent the function of VEGFR-2 under EGF stimulation (22)). Second, there was no effect on EGF-stimulated HUVEC migration when HUVECs were cotransduced with EGNP-1 and EGDR mutants or EGLT mutants. EGDR(Y951F) is a mutant of EGDR in tyrosine residue 951 of VEGFR-2 that completely abolishes the EGF (up to 100 ng/ml)-induced migratory activity in HUVECs transduced with EGDR(Y951F)/(Fig. 3a, D) (24). Finally, blocking VEGFR-2 with an anti-VEGFR-2 antibody on EGNP-1-transduced HUVECs had no effect on EGF-induced HUVEC migration. Overall, these results indicate that NRP-1/EGNP-1 can mediate ligand-dependent HUVEC migration as an independent receptor. This finding is supported by a number of previous studies. Neuropilin binding with VPF/VEGF isoform enhanced breast carcinoma cell (lacking the expression of VEGFR-2) survival by stimulating the PI3K pathway (14). However, the present study seems to contradict our previous result (4) that showed no migration response to VGF, when NRP-1 alone was expressed on porcine aortic endothelia cells. The possible explanation is that porcine aortic endothelia cells do not normally express detectable levels of VEGFR-2, VEGFR-1, or NRP-1 and do not respond to VPF/VEGF (4, 36). Therefore, porcine aortic endothelia cells may lack related signaling molecules with the absence of a receptor and may be less representative of vascular endothelium than the early passaged HUVECs used in this study. On the other hand, VEGFR-2 bound to VEGF165 more efficiently in cells expressing NRP-1, and this potentiating effect was subsequently translated into a better migratory response to VEGF165 as compared with the migratory response of cells expressing VEGFR-2 but not NRP-1. Thus, NRP-1 seems to function as an enhancer of VEGFR-2 activity in the presence of VEGF165. This effect is probably because of a complex formation between VEGFR-2 and NRP-1 (12).

This study is the first report that demonstrates the C-terminal three amino acids of NRP-1 (SEA-COOH) are essential for NRP-1-mediated HUVEC migration. It is known that the C-terminal three amino acids of NRP-1 (SEA-COOH) are responsible for interaction with the PDZ domain-containing C-terminal two-thirds of NRP-1-interacting protein (NIP) (20, 21). One of the important roles of PDZ domain-containing proteins is to act as molecular adapters that target proteins to proper subcellular compartments or assemble signal transduction components into closely associated protein complexes (37–39). The physical interaction and co-localization of NRP-1 and NIP in the nervous system suggested that NRP-1 functionally interacts with NIP (20). Recently, NIP has been independently cloned as RGS-GAIP-interacting protein, where it was identified by virtue of its interaction with the C terminus of RGS-GAIP (a Gαi3-associated protein located to the membrane of clathrin-coated vesicles) and was suggested to participate in the regulation of clathrin-coated vesicular trafficking by association with the G protein-coupled signaling complex (21). In view of these findings as well as ours, we postulate that there may be a PDZ domain-containing NIP in HUVECs that participates in the regulation of NRP-1-mediated migration signaling by interacting with the C-terminal three amino acids of NRP-1 (SEA-COOH).

The involved signaling molecules for NRP-1/EGNP-1-mediated HUVEC migration were investigated in this study. We found that PI3K inhibitor Ly294002 and the dominant negative mutant of p85 (p85DN) inhibit EGNP-1-mediated HUVEC migration induced by EGF, but the constitutive activated mutant of PI3K (p110CaAX) expression results in more migration. These observations indicate that PI3K is involved in the NRP-1-mediated migration signaling pathway, which is consistent with the findings by Bachelor et al. (14) that neuropilin activates the PI3K pathway induced by VPF/VEGF in metastatic breast carcinoma cells which is important for the survival of these cells (14). The experiments in G proteins showed that Gq antagonist 2A, a pharmacological antagonist of Gαq proteins, causes almost complete inhibition of EGNP-1-mediated HUVEC migration induced by EGF, and further showed that Gβγ-sequestering peptide hARK (495) significantly inhibited EGNP-1-mediated HUVEC migration. These data demonstrate that Gαq family proteins are involved in VPF/VEGF-stimulated NRP-1-mediated HUVEC migration, and Gβγ subunits are required for this signaling pathway. In corroboration with the previous study in our laboratory that heterotrimeric G protein Gαq/11 and Gβγ subunits are required for VEGF2-mediated EC migration (23), this study further confirms that Gαq family proteins and Gβγ subunits are required for VPF/VEGF-stimulated EC migration. The current study found that the RhoA dominant negative mutant, RhoA-19N, significantly inhibits EGF-stimulated EGNP-1-mediated HUVEC migration. This indicates that the small GTPase RhoA is involved in the VPF/VEGF-induced NRP-1-mediated EC migration signaling pathway and again suggests that the effect of VPF/VEGF on EC mobility is mediated through the RhoA pathway at least in early passaged HUVECs (23). This conclusion is not in conflict with Liu et al. (40), who find that overexpression of a dominant negative Rho did not inhibit VPF/VEGF-stimulated bovine pulmonary artery endothelial cell migration (40). Possible reasons for the apparent inconsistency are that arterial ECs may have different physiological phenotypes (41) and that the early passaged (passage 3–4) HUVECs in our experiments may have a different pattern of expression of VPF/VEGF receptors from bovine pulmonary artery endothelial cells of passage 19–24 used in their experiment. Because Ly294002 (a PI3K inhibitor) inhibits the VEGF-1 signaling and, therefore, inhibits VPF/VEGF-stimulated migration of late passaged bovine pulmonary artery endothelial cells (42), it is likely that VEGF-1 may mediate VPF/VEGF-induced migration of late passaged bovine pulmonary artery endothelial cells. NRP-1 stimulation leading to RhoA-dependent HUVEC migration was further identified by a RhoA activation assay in this study. With the results of pull-down assays we found that EGNP-1 mediated EGF-induced RhoA activation in HUVECs, but the mutant of EGNP-1, EGNP-1-SEA, did not show the mediatory function of EGNP-1. Furthermore, we demonstrated the PI3K inhibitor (Ly294002) and the dominant negative mutant p85DN could inhibit this activation. Pretreatment with an antagonist of Gαq family proteins, Gp antagonist 2A, inhibits EGNP-1-mediated RhoA activation, which further confirms the involvement of Gαq family proteins in NRP-1 signaling; Gβγ-sequestering peptide hARK (495) inhibits EGNP-1-induced activation, indicating that Gβγ is the upstream mediator of NRP-1 signaling to RhoA activation. Taken together, these results demonstrate that NRP-1-mediated RhoA activation requires Gαq family proteins, free Gβγ subunits, and PI3K activation. Overall, our current model for NRP-1-stimulated leads to EC migration is VPF/VEGF → NRP-1 → PDZ domain-containing protein → Gαq family proteins → Gβγ subunits → PI3K → RhoA → EC migration. Our future studies will be aimed at elucidating more complete
mechanisms of NRP-1 functional activity and identifying the functional activities in vivo.

A major contribution of this study is the findings that NRP-1 alone can mediate VPF/VEGF-induced EC migration, that the intracellular domain of NRP-1 is involved in this function, and that the C-terminal three amino acids are essential for this function. As invasion and metastatic spread is viewed as angiogenesis-dependent events (28), regulating EC migration and tumor cell migration as well as spread have drawn considerable attention from investigators interested in cancer control. The finding that NRP-1 alone can mediate EC migration along with the fact that NRP-1 is not only expressed in EC but also in metastatic tumor cells (4) suggests that NRP-1 may be an important determinant of metastasis. Therefore, this study holds significant implications both in understanding the mechanisms of tumor cell metastasis and potentially in targeting NRP-1 for anti-cancer therapy.

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J. Biol. Chem. 2003, 278:48848-48860.
doi: 10.1074/jbc.M310047200 originally published online September 26, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M310047200

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