Inhibition of Tumor Cell Migration by LD22-4, an N-Terminal Fragment of 24-kDa FGF2, Is Mediated by Neuropilin 1

Ling Zhang1, Graham C. Parry2, and Eugene G. Levin1,2

Abstract

LD22-4, an 86-amino acid fragment of the basic fibroblast growth factor, is an inhibitor of cell migration. LD22-4 inhibits the migration of various tumor cells, endothelial cells, and fibroblasts in vitro and suppresses tumor growth and angiogenesis in vivo. LD22-4 is effective in the presence of multiple growth factors, either alone or in combination, as well as haptotactic factors. LD22-4 inhibits the rate of malignant gliomas prepared from U87MG cells in an orthotopic mouse model by 90% compared with untreated mice. Using U87MG cells, we identified the LD22-4 membrane receptor as neuropilin 1 (NRP1). The identification of NRP1 as the LD22-4 receptor was based upon mass spectrometric analysis of proteins that bind to LD22-4, immunoprecipitation of an NRP1–LD22-4 complex formed during incubation of LD22-4 with U87MG cells, LD22-4–NRP1 coimmunoprecipitation studies, and binding of LD22-4 to HEK293 cells expressing NRP1. In contrast, NRP1 binding of an inactive mutant of LD22-4 was substantially reduced. As is typical of NRP1-binding proteins, LD22-4 itself binds to heparin and requires heparan sulfate for binding to cells. The addition of heparin to migration assays increased the inhibitory activity of LD22-4. In addition to a heparin-binding region, LD22-4 contains a 5-amino acid C-terminus that matches an NRP1 consensus binding sequence. Thus, direct binding experiments, dependence on heparan sulfate, and the presence of an NRP1 consensus binding sequence indicate that NRP1 is the binding site of LD22-4 and mediates inhibition of cell migration. Cancer Res; 73(11); 3316–25. ©2013 AACR.

Introduction

LD22-4 (previously referred to as ATE+31), an 86-amino acid N-terminal fragment of the basic fibroblast growth factor (bFGF2), is an inhibitor of cell migration (1, 2). LD22-4 inhibits the migration of tumor cells, endothelial cells, and fibroblasts in vitro and is effective in the presence of various growth factors that promote enhanced migration [VEGF, insulin-like growth factor (IGF), EGF, platelet-derived growth factor (PDGF)], complex mixtures of growth factors and cytokines (serum), as well as haptotactic factors (fibronectin). LD22-4 suppresses tumor growth and angiogenesis by more than 90% in animal models using breast, prostate, and lung carcinoma-derived tumors (1, 2). LD22-4 is not cytotoxic, does not induce apoptosis, and does not have a significant effect on proliferation rates of endothelial cells and tumor cells. LD22-4 has a highly basic N-terminal domain containing 3 clusters of 3 arginines each, and arginine to alanine substitution in the amino terminal 2 clusters diminishes LD22-4 activity. Mechanistically, LD22-4 blocks focal adhesion kinase (FAK) phosphorylation in response to growth factors, specifically the phosphorylation of Tyr107–FAK and Ser392–FAK (3). FAK-tyr607 is required to recruit paxillin and vinculin to FAK and to ensure formation of focal adhesions (4). LD22-4 does not affect the phosphorylation of FAK-tyr961, tyr925, tyr577, or tyr397 nor the phosphorylation of PYK2, src kinases, Erk1/2, or AKT following growth factor treatment (3–5). Thus, LD22-4 seems to target a highly specific phosphorylation event necessary for cell migration. The failure of growth factors to phosphorylate FAK-tyr607 in the presence of LD22-4 occurs simultaneously with the failure of FAK, focal adhesion plaques, and actin stress fibers to redistribute within the cell cytoplasm and periphery, suggesting that LD22-4 causes a systemic failure in the mechanisms promoting cell migration (3).

Neuropilin 1 (NRP1) is a single-pass transmembrane glycoprotein with multiple ligands (6–8). The primary role of NRP1 is the regulation of cell motility, particularly with respect to neural and vascular development (7–12). NRP1 forms a co-receptor complex with VEGFR2 through the bridging of the 2 receptors by VEGF (6, 9, 13–16). NRP1–VEGFR1–VEGFR2 complex formation leads to enhanced VEGFR2 activation, actin reorganization, and the stimulation of cell migration, and blocking VEGF binding to NRP1 diminishes the rate of cell migration although it has no effect on cell growth (9, 17). Thus, NRP1 has been associated with the VEGF-dependent stimulation of cell migration. Blocking VEGF binding to NRP1 does not affect VEGF-induced phosphorylation of Erk1/2 or Akt, indicating that NRP1 is not required for the activation of all of the VEGF signaling pathways and that some occur exclusively through VEGFR–VEGF interaction (17). In addition to VEGF, NRP1 also interacts with other growth factors including bFGF2, hepatocyte growth factor (HGF/SF), PDGF, and...
placental growth factor (PIGF; refs. 10, 18–22). The NRP1-binding sites for bFGF2 and HGF are distinct from that of VEGF; an antibody that blocks VEGF binding to NRP1 and does not interfere with cell migration promoted by FGF2 or HGF (22), NRP1 is also required for p130Cas phosphorylation in response to HGF and PDGF in malignant glioma cells (10).

NRP1 is expressed by human tumor cell lines and tumor cells derived from lung, breast, prostate, pancreatic, and colon carcinomas, but is not found in the corresponding normal tissues (7, 17, 23–26). Clinical studies suggest that NRP1 plays a role in tumor growth and disease progression. It is preferentially expressed in metastatic cells, and is associated with invasive behavior and metastatic potential. Overexpression of NRP1 in prostate and colon cancer cells enhances angiogenesis and tumor growth in animals. We present here evidence that NRP1 acts as the receptor for LD22-4, and that the binding characteristics and subsequent effects are consistent with those defined for protein–NRP1 interaction.

Materials and Methods

Cell culture

U87MG and HEK293 cell lines were purchased from the American Type Culture Collection and cultured according to the supplier’s instructions. U87MG cells expressing luciferase (U87MG-luc) were provided by Dr. Patrick McConville (Molecular Imaging, Inc.). The cell lines used in this study have not been tested or authenticated.

Purification and biotinylation of LD22-4

LD22-4 was prepared as described previously (2). Biotinylation of LD22-4 was carried out with 0.8 mg/mL LD22-4 in the presence of 5 mmol/L Sulfo-NHS-LC-Biotin (EZ-Link Sulfo-NHS-LC-Biotin; Pierce) with PBS for 1 hour at room temperature. The reaction was quenched by the addition of 100 mmol/L Tris–HCl, pH 7.4, and the reaction mixture was dialyzed against PBS. LD63-6, a functionally deficient mutant of LD22-4 in which the arginines within the first 22 amino acids are converted to alanines, was prepared and biotinylated in the same way. Both LD22-4 and LD63-6 were generated with a His-tag inserted at the C-terminal end to allow for immunodetection with equal avidity.

Migration assay

Cell migration assays were conducted using modified Boyden chambers with a 6.5-mm diameter and a 8.0 μm pore size polycarbonate membrane separating the 2 chambers (Nalge Nunc International) as described previously (1). Both sides of the membrane were coated with vitronectin at 1 μg/mL for 2 hours at room temperature. The results were expressed as a percentage of the number of cells migrating in the presence of PDGF alone ± SD. All experiments were conducted in triplicate.

Binding experiments

U87MG cells (5 × 10⁴ cells) were plated on glass coverslips (18 mm) coated with poly-L-lysine (Sigma) incubated with 2.5 μmol/L biotinylated LD22-4 or biotinylated LD63-6 in PBS, fixed in 4% (w/v) paraformaldehyde, and incubated with fluorescein isothiocyanate (FITC)–streptavidin.

Fractionation of U87MG plasma membrane and isolation of LD22-4–binding proteins

U87MG glioma cells were disrupted by Dounce homogenization, and centrifuged at 800 × g for 20 minutes to remove the nuclear fraction. The remaining cell lysates underwent centrifugation at 45,000 × g for 20 minutes at 4°C, and the pellet was resuspended in 1 mL of lysis buffer containing 1% octyl glucoside (OG) for 2 hours at 4°C. The remaining insoluble material was removed by centrifugation at 45,000 × g for 20 minutes at 4°C. To isolate LD22-4–binding proteins, LD22-4 or LD63-6-Sepharose was incubated with 100 μL U87MG membrane extract for 2 hours at 4°C. The Sepharose–protein complex was removed from the reaction mixture by centrifugation, and treated with 5 mmol/L β-mercaptooctanol and mass spectroscopy (MS)-grade trypsin (Invitrogen) overnight at 37°C. Sepharose beads were removed by centrifugation and the digest was analyzed by nano-liquid chromatography/tandem mass spectrometry (LC/MS-MS) on an LTQ Mass Spectrometer at The Scripps Research Institute Center for Mass Spectrometry. The raw data were screened using the MASCOT NCBI Homo Sapiens database.

Generation of HEK293 cells expressing NRP1

HEK293 cells were transfected with a plasmid consisting of the full-length human NRP1 cDNA in a pENTR221 mammalian expression vector (GeneCopoeia, Inc.).

LD22-4–NRP1 coimmunoprecipitation analysis

Binding of LD22-4 to NRP1 was analyzed by 3 methods: (i) U87MG cells were treated with 2.5 μmol/L LD22-4 or LD63-6 for 10 minutes at 37°C, washed with cold PBS twice, and lysed with immunoprecipitation buffer containing 1% OG plus protease inhibitors. Equal amounts of protein were treated with anti-neuropilin-1 antibody overnight at 4°C, the immune complexes were precipitated with protein A/G agarose (Calbiochem), and samples were analyzed by SDS-PAGE and immunoblotting with biotinylated anti-His antibody; (ii) Cell membrane extracts were prepared and 2.5 μmol/L LD22-4–Sepharose or LD63-6-Sepharose was incubated with 100 μL U87MG membrane extract for 4 hours at 4°C. The complexes were removed by centrifugation and analyzed by SDS-PAGE and immunoblotting with α-NRP1 antibodies; (iii) U87MG cell membrane extracts were prepared and 100 μL incubated with α-NRP1-agarose conjugates overnight at 4°C. The NRP1–α-NRP1-agarose was incubated with 2.5 μmol/L LD22-4 or LD63-6 for 4 hours at 4°C. Complexes were precipitated by centrifugation and analyzed by SDS-PAGE and immunoblotting with α-His and α-NRP1 antibodies.

In vivo analysis of LD22-4 activity

All experiments were conducted with 6- to 7-week-old CD-1 nu/nu female mice. All procedures carried out in this experiment were conducted in compliance with all the laws, regulations, and guidelines of the National Institutes of Health (NIH) and with the approval of the Institutional Animal Care and Use
Committee. Each animal underwent surgery for implantation of a stainless steel cannula (13-mm long and 0.635 mm in diameter) 3 mm into the right frontal brain. Animals were allowed to recover from surgery for 1 week before tumor cell inoculation. Mice were treated with 0, 0.44, or 4.4 μg LD22-4 every 4 days and bioluminescence imaging (BLI) was carried out every 7 days.

Results

Inhibition of glioma migration and tumor growth by LD22-4

LD22-4 inhibited U87MG cell migration in a dose-dependent manner with maximum inhibition (26.2 ± 8.5% residual activity) occurring at 2.5 μmol/L (Fig. 1A). No further inhibition occurred at higher concentrations of LD22-4. This maximum inhibition is consistent with that observed with all cell types and chemoattractants in previous studies (1–3). LD 63-6, the LD22-4 mutant containing arginine to alanine substitutions in the N-terminus (1), was less effective in inhibiting cell migration than LD22-4, reducing migration by only 20% (to 80.3 ± 7.8% of control cultures) at the same concentration (Fig. 1B).

The effect of LD22-4 on the growth of malignant gliomas was tested in an orthotopic mouse model. Animals were treated with 0, 0.44 (17.6 μg/kg), or 4.4 μg (176 μg/kg) LD22-4 (Fig. 2A) and tumor growth was measured by BLI every 7 days (arrows). In all 3 cohorts, tumor growth remained static for 20 days followed by a comparable increase from 20 to 27 days (Fig. 2A). Tumor size continued to increase in the untreated and 0.44 μg LD22-4–treated animals, but animals treated with 4.4 μg LD22-4 showed no further increase in mean BLI intensity; at day 35, the average tumor size in these animals was only 21 ± 4% that of the control group (P = 0.016). Tumors in the group treated with 0.44 μg were 65 ± 16% that of the untreated group although this decrease was not statistically significant (P = 0.29). The group medians were 13% and 84% of the control group in the animals treated with 4.4 and 0.44 μg LD22-4, respectively (Fig. 2B). The experiment was repeated with 4.4 μg LD22-4 and similar results were observed (Fig. 2C); at day 35,
the tumors were 10 ± 2% (group mean; P = 0.017) or 9% (group median) of those in the untreated group. No treatment-related deaths occurred during the study, and changes in the weights of control and LD22-4–treated animals were similar (data not shown). Histologic analysis of the brains at day 35 confirmed the BLI results (Fig. 2D); 4.4-μg LD22-4–treated animals showed only traces of residual tumor (circled area) compared with the large tumors present in the brains of untreated mice.

**LD22-4 binding to U87MG cells**

U87MG cells were incubated with 2.5 μmol/L biotinylated LD22-4 in the presence or absence of unlabeled LD22-4 (Fig. 3). LD22-4 was uniformly distributed throughout the cell surface (Fig. 3A). To verify that binding was specific, unlabeled LD22-4 at a 20- (Fig. 3B) and 60-fold (Fig. 3C) molar excess was included in the binding solution. A 20-fold excess of unlabeled LD22-4 significantly reduced fluorescence intensity, whereas little detectable fluorescence remained at 60-fold excess. In contrast, binding of LD63-6 was significantly reduced, but not completely eliminated (Fig. 3D, control; 3E, LD63-6). No binding was observed with trypsin-treated biotinylated LD22-4 indicating that the positive signals were not due to the biotinylation of cell surface molecules by residual biotin reagents (data not shown).

**Identification of the LD22-4–binding protein**

Isolation of an LD22-4 cell surface-binding protein was carried out by subjecting U87MG cell membrane extracts to affinity chromatography on LD22-4-Sepharose and LD63-6-Sepharose, digestion of bound proteins trypsinization, and the digest analyzed by MS-based proteomics. Among the proteins binding specifically to LD22-4, only 1 was identified as a known surface receptor with a transmembrane sequence and a
biologic role in the regulation of cell migration, NRP1. Table 1 lists the peptides corresponding to NRP1 that were obtained when the LD22-4-binding proteins were subjected to MS.

Confirmation that LD22-4 specifically bound to NRP1 was obtained with HEK293 cells expressing human NRP1 (HEK293-NRP1; Fig. 4). Expression of NRP1 by the HEK293-transfected cells was validated by fluorescence-activated cell sorting (FACS) analysis of cells transfected with the NRP1 gene (Fig. 4B, α-NRP1). For LD22-4-binding studies, 0, 100, and 1,000 nmol/L biotinylated LD22-4 were incubated with HEK293 or HEK293-NRP1; no binding of LD22-4 was observed at 100 nmol/L, whereas 1,000 nmol/L LD22-4 caused a shift in fluorescence, showing that the presence of NRP1 on the cell surface promotes LD22-4 binding. Binding of LD22-4 to HEK293-NRP1 but not HEK293 parental cells was also shown by immunohistochemical analysis and fluorescent microscopy (Fig. 4A).

Further evidence that LD22-4 binds to NRP1 was sought by coimmunoprecipitation experiments using U87MG membrane NRP1. Three approaches were taken: (i) LD22-4-Sepharose or LD63-6-Sepharose was incubated with U87MG cell membrane extracts, and the presence of NRP1 was evaluated by immunoblotting (Fig. 5A). NRP1 in crude membrane extracts appeared as a doublet with a top band representing a molecular weight of 130 kDa, the defined molecular weight of NRP1, and a lower band of approximately 110 kDa. NRP1 has also been observed as a doublet in extracts of U87MG cells, smooth muscle cells, endothelial cells, and tissue removed from glioma tumors in previous studies (10, 19, 27). No explanation of the source of the lower band has been presented, and it is unclear whether it is the result of proteolytic cleavage or regulated transcriptional or translational modification. Immunoblot analysis of LD22-4- and LD63-6-Sepharose-binding proteins showed NRP1 had associated with both, although a much smaller amount was observed in samples containing LD63-6-Sepharose (Fig. 5A). However, only the top NRP1 band of the doublet was observed in both samples. As it is not clear whether the difference in molecular weight is due to protein size or posttranslational modification, the apparent difference in affinity cannot be explained. (ii) Formation of an NRP1-LD22-4 complex was evaluated by isolating NRP1 from U87MG membrane extracts with α-NRP1-agarose and then incubating the immune complex with LD22-4 or LD63-6 (Fig. 5B). Immunoblot analysis of the resulting agarose-α-NRP1-associated proteins revealed the presence of LD22-4. LD63-6 was also detected, but at lower levels than LD22-4. To ensure that the bound NRP1 was presented to LD22-4 and LD63-6 in equal amounts, aliquots of agarose-α-NRP1 were removed after incubation with the membrane extracts and the relative amount of NRP1 was determined. The results showed little difference in the amount of NRP1 available to LD22-4 and LD63-6 (Fig. 5B, right). (iii) Direct cell surface binding experiments were conducted by incubating LD22-4 or LD63-6 with

Table 1. NRP1 peptides obtained during mass spectrometry of LD22-4-binding proteins

<table>
<thead>
<tr>
<th>Mr (exp)</th>
<th>Mr (calc)</th>
<th>Expect</th>
<th>Score</th>
<th>Peptides</th>
<th>Neurepi1l 1</th>
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<td>1897.4258</td>
<td>1896.8278</td>
<td>2.8 × 10^-4</td>
<td>65</td>
<td>R.LNYPENGWTPGEDSYRE.E</td>
<td>307 RNYPENGWTPGEDSYRE 324</td>
</tr>
<tr>
<td>2090.3026</td>
<td>2088.8813</td>
<td>0.24</td>
<td>35</td>
<td>K.YDYYVEVFDBEGHFR.G</td>
<td>83 KYDYVEVFDBEGHFRG 101</td>
</tr>
<tr>
<td>2535.9037</td>
<td>2534.1310</td>
<td>1.4 × 10^-4</td>
<td>67</td>
<td>K.TGPIQDHGTDGGNFYQSADENQKG</td>
<td>679 KTGPIQDHGTDGGNFYQSADENQKG 703</td>
</tr>
</tbody>
</table>

NOTE: M_r (exp), observed peptide mass; M_r (calc), calculated peptide mass; Expect value, frequency of this match occurs by chance, <0.05 = statistical confidence at the 5% threshold; Score, ion score.
intact U87MG cells and isolating the complexes after cell extraction by immunoprecipitation with α-NRP1-agarose (Fig. 5C). LD22-4 was present, whereas LD63-6 was barely detectable (left). Comparison of the amount of NRP1 captured by α-NRP1-agarose in extracts containing LD22-4 or LD63-6 showed that they were equal.

LD22-4 binds heparin

Heparin plays a crucial role in the interaction between NRP1 and its binding proteins. VEGF, FGF2, PDGF, and HTLV all bind to heparin and removal of heparan sulfates from the cell surface limits the binding of the proteins to NRP1 (21, 22, 28, 29). To determine whether LD22-4 has an affinity for heparin,
Binding experiments were conducted with LD22-4 and heparin-agarose (Fig. 6). Under physiologic salt concentrations, LD22-4 bound to heparin-agarose and was only partially eluted with 350 mmol/L NaCl. Complete elution of the protein from heparin required 500 mmol/L NaCl (Fig. 6A). Elution of LD22-4 from the heparin-agarose could also be achieved with free heparin with 50% removal at 125 μg/mL heparin and complete removal at 625 μg/mL heparin (Fig. 6B, left). Little binding of LD63-6 to heparin was observed (Fig. 6B, right), suggesting that the arginine-rich N-terminus of LD22-4 is required for heparin binding. Heparinase treatment before addition of the protein resulted in a decline in LD22-4 binding, which was not further reduced with additional lyase. To determine whether cell surface heparan sulfate was required for LD22-4 binding to U87MG cells, cells were treated with heparitinase I (Fig. 6C and D). LD22-4 binding was reduced but not completely eliminated. Immunologic detection showed that heparan sulfate could not be detected on the cell surface following digestion (Fig. 6E). Finally, to metabolically block the sulfation of GAGs, cells were treated with sodium chlorate for 20 hours and binding experiments repeated. Incubation with 20 mmol/L sodium chlorate reduced LD22-4 binding, although some residual binding could be detected.
be observed (Fig. 6F). Cell viability following sodium chlorate treatment was 85 ± 6%.

The functional effect of heparin on LD22-4 activity was assessed by treating glioma cells with LD22-4 in the presence or absence of 10 μg/ml heparin (Fig. 6G). Heparin itself at a concentration of up to 625 μg/ml had no effect on U87MG cell migration (data not shown). However, when cells were treated with 2.5 μmol/L LD22-4 and 10 μg/ml heparin, the rate of cell migration declined from 17.5 ± 6.6% to 6.6 ± 4.3% of untreated cultures.

Discussion

In this report, we identify NRP1, a growth factor coreceptor linked to the control of cell migration, as a cell surface binding site for LD22-4. This conclusion is supported by MS analysis of proteins that bind to LD22-4, immunoprecipitation of an NRP1–LD22-4 complex formed during incubation of U87MG cells with LD22-4, coimmunoprecipitation of the 2 proteins, and binding of LD22-4 to HEK293 cells expressing NRP1.

There are several characteristics of LD22-4 that are consistent with a protein that binds to NRP1. One of the most striking is the C-terminal sequence of LD22-4. Studies using phage display screens to identify a class of peptides that induce cell binding and internalization showed that those with the greatest binding affinity had a terminal consensus R/KXXR/K sequence (30). Most efficient were peptides with a C-terminal arginine residue preceded by an arginine or lysine. Optimum binding affinity occurred when 2 non-basic amino acids separated the R/K at either end of the consensus sequence. For example, a peptide containing the sequence TKPR (enhanced avidity than the peptide TKPR (tuftsin; ref. 31). NRP1 was identified as the receptor for the peptides containing this motif. VEGF$_{165}$ interacts with NRP1 through a C-terminal consensus-like sequence (CRDCDKPR) and tuftsin, enhanced tuftsin, and A7R (ATWLPPR), compete with VEGF$_{165}$ for NRP1-binding (31–33). This occurred in tumor cell lines, vascular endothelial cells, and in cells isolated from normal mouse organ. In addition, this consensus sequence is found within the HTLV surface unit, and is required for virus binding to NRP1. The C-terminus of LD22-4, KDPKR, is identical to the consensus binding sequence defined by this class of NRP1-binding proteins. Thus, it is likely that LD22-4 is a member of a class of proteins that binds to NRP1 through this specific sequence. In fact, deletion mutagenesis studies showed that the absence of the C-terminal 11 amino acids of LD22-4 (which includes the KDPKR sequence) reduced the inhibition of cell migration by half. Therefore, we propose that maximum binding of LD22-4 to NRP1 is dependent on both the C-terminal sequence and the heparin-binding region.

Heparin binding to growth factors and NRP1 itself is an essential part of NRP1 interaction, dimerization, and signal transduction (6, 21, 22, 32, 34). Both VEGF$_{165}$ and NRP1 bind heparin and heparan sulfate that enhance the interaction between the 2 proteins by 100-fold. LD22-4–heparin-binding studies show that the protein has an affinity for heparin that withstands salt concentrations greater than physiologic levels, and requires an excess of heparin for dissociation. The reduction of LD22-4 binding following heparinase digestion of the cell surface and the increased inhibition of migration in the presence of added heparin indicates that LD22-4–heparin binding has a functional consequence leading to increased LD22-4 activity. Heparin sulfates on the cell surface also seem to play some role in LD22-4 binding because enzymatic digestion by heparitinase reduces LD22-4 binding. The apparent difference in binding of LD22-4 following digestion with each lyase is probably due to differing requirements for the specific GAGs affected by the enzymes. In both cases of GAG degradation, LD22-4 binding is not completely eliminated, suggesting that there are multiple factors required for optimum binding. The NRP1 C-terminal consensus binding sequence is a likely candidate for a secondary binding site that acts in concert with the GAGs.

The mechanism by which LD22-4 blocks cell migration through NRP1 binding remains to be elucidated. Simple competition for growth factor binding to NRP1 would involve LD22-4 blocking the interaction of multiple growth factors, which do not bind to the same locus on NRP1. In addition, neither IGF-I nor fibronectin have been found to bind to NRP1. An alternative to directly interfering with growth factor binding is blocking the transmission of signals that promote migration. Proteins involved in the formation of signaling receptor complexes are often single transmembrane domain proteins that form homo- or heterodimers and oligomeric complexes that are crucial for signal transduction (36–38). NRP1 fits this description and has a putative dimerization motif found in the transmembrane domain (39, 40). If NRP1 signaling is dependent on the formation of NRP1 dimers or oligomers as this model predicts, then LD22-4 could disrupt NRP1 function by interfering with NRP1 dimerization or its interaction with other membrane receptors. Finally, the interaction between LD22-4 and NRP1 may directly interfere with signal transduction by suppressing the association of NRP1 with cytoplasmic proteins that initiate intracellular signaling pathways. There are also studies that suggest that NRP1 does not necessarily function solely as a coreceptor, but can signal independently of growth factor receptor interaction through NRP1 cytoplasmic domain interaction with nonreceptor intracellular proteins (8, 10, 16, 41, 42). Deletion mutagenesis studies show that the 3 amino acids in the C-terminal, serine–glutamic
acid–alanine, are essential for NRP-1–mediated HUVEC migration and that this involves the RGS–GAP–interacting protein that binds to NRP1 through the SEA-COOH motif (16, 43). Thus, another mechanism by which LD22–4 may inhibit migration is through interference or disruption of interaction between the NRP1-cytoplasmic domain and intracellular proteins.

Taken together, the data presented in this study are consistent with NRP1 acting as the cell surface receptor for LD22–4. The fact that NRP1 regulates cell migration is also consistent with it being the target of a protein such as LD22–4 that acts as an inhibitor of cell motility. The efficacy of LD22–4 in the presence of various growth factors and the identification of NRP1 as the target for LD22–4–cell interaction suggest that there is a binding site on NRP1 for LD22–4 that can be exploited to inhibit cell migration regardless of its stimulus. Identification of the precise binding site on NRP1 and the mechanism by which it effects migration will allow for development of more efficacious compounds to control tumor growth and angiogenesis.

References


Disclosure of Potential Conflicts of Interest

E.G. Levin has ownership interest (including patents) in Motility Incorporated. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: E.G. Levin

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): I. Zachary, E.G. Levin

Writing, review, and/or revision of the manuscript: L. Zhang, G.C. Parry, E.G. Levin

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E.G. Levin

Study supervision: L. Zhang, G.C. Parry, E.G. Levin

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