Determination of Cell Adhesion Sites of Neuropilin-1

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Abstract. Neuropilin-1 is a type I membrane protein with three distinct functions. First, it can mediate cell adhesion via a heterophilic molecular interaction. Second, in neuronal cells, neuropilin-1 binds the class 3 semaphorins, which are neuronal chemorepellents, and plays a role in the directional guidance of axons. Neuro- pilin-1 is expected to form complexes with the plexinA subfamily members and mediate the semaphorin-elicited inhibitory signals into neurons. Third, in endothelial cells, neuropilin-1 binds a potent endothelial cell mitogen, vascular endothelial growth factor (VEGF)165, and regulates vessel formation. Though the binding sites in neuropilin-1 for the class 3 semaphorins and VEGF165 have been analyzed, the sites involved in cell adhesion activity of the molecule have not been identified. In this study, we produced a variety of mutant neuropilin-1s and tested their cell adhesion activity. We showed that the b1 and b2 domains within the extracellular segment of neuropilin-1 were required for the cell adhesion activity, and peptides with an 18-amino acid stretch in the b1 and b2 domains were sufficient to induce the cell adhesion activity. In addition, we demonstrated that the cell adhesion ligands for neuropilin-1 were proteins and distributed in embryonic mesenchymal cells but distinct from the class 3 semaphorins, VEGF, or plexins.

Key words: neuropilin-1 • cell adhesion • cell aggregation • semaphorins • mutant protein

Introduction

The identification and characterization of the cell surface receptor proteins that help to trigger the intercellular signals to regulate cell proliferation, differentiation, migration, or cell–cell contact are important if one is to understand the cellular and molecular mechanisms underlying the organization of multicellular tissues or organs.

Neuropilin-1 is a unique membrane protein that is highly conserved among various vertebrate species, including Xenopus frog (Takagi et al., 1987, 1991), chicken (Takagi et al., 1995), mouse (Kawakami et al., 1996), rat (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997), and human (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). Neuropilin-1 is expressed in a variety of neuronal and non-neuronal cells and can mediate at least three distinct intercellular signals to regulate diverse aspects of embryonic development.

First, neuropilin-1 functions as a cell adhesion receptor. When neuropilin-1 is expressed on cell surfaces of the fibroblast cell line, L cells, it interacts with some unknown ligand(s) on the surface of the cells and mediates cell–cell adhesion (Takagi et al., 1995). Second, neuropilin-1 is expressed in particular classes of neurons (Takagi et al., 1987, 1991, 1995; Kawakami et al., 1996), and binds the class 3 semaphorins (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997), which are potent neuronal chemorepellents (Luo et al., 1993; Kolodkin et al., 1993; Fan and Raper, 1995; Messersmith et al., 1995; Püschel et al., 1995). We have reported that, in neuropilin-1-deficient mutant mice produced by targeted disruption of the neuropilin-1 gene, neuropilin-1-deprived dorsal root ganglion neurons are protected from growth cone collapse elicited by semaphorin 3A (Sema3A/SEMA3A; previously, collapsin-1/semaphorin D/semaphorin III; Semaphorin Nomenclature Committee, 1999), and their fibers were misguided (Kitsukawa et al., 1997). These results indicate that neuropilin-1 functions as a receptor or an indispensable component of receptor complex for the class 3 semaphorins to transduce semaphorin-elicited repulsive signals into growth cones (Chen et al., 1997; Feiner et al., 1997; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). More recently, it has been shown that neuropilin-1 forms complexes with other neuronal membrane protein, plexins, and propagates Sema3A signals (Takahashi et al., 1999; Tamagnone et al., 1999).
Third, neuropilin-1 is expressed in endothelial cells (Kit- sukawa et al., 1995), and binds an isofrom of vascular end- othelial growth factor (VEGF)1, VEGF165 (Soker et al., 1998), which is a major regulator of vascular-angiogenesis (Ferrara and Henzel, 1989; K ech et al., 1989; Leung et al., 1989). O verexpression of neuropilin-1 in mouse embryos resulted in an excess production of blood vessels and mal- formed hearts (Kit-sukawa et al., 1995). In contrast, the neuropilin-1-deficient mouse embryos exhibited severe defects in embryonic vessel formation (K awasaki et al., 1999). These findings indicate that neuropilin-1 interacts with VEGF165, and plays important roles in vasculo-angiogenesis.

The extracellular segment of neuropilin-1 has a domain combination different from that of any other cell surface receptor, consisting of five domains known as a1, a2, b1, b2, and c, each of which is shared by a wide variety of mol- ecules (Takagi et al., 1991, 1995; K awakami et al., 1996). The a1/a2-like domains are shared by the complement components C1r and C1s (Leytus et al., 1986; Mac kinnon et al., 1987), the human bone morphogenetic protein-1 (B MP-1; W ozney et al., 1988), the Drosophila dorsal-ven- tral patterning protein Tolloid (Shimell et al., 1991), and the choroid plexus protein p14 (Le cain et al., 1991). The b1/b2-like domains exist in the coagulation factors V and V Ill (Toole et al., 1984; J enny et al., 1987), a protein com- ponent of the fat globule membrane (M FGPs; Stubbs et al., 1990), and receptor tyrosine kinase DDR (the discoidin domain receptor; J ohnson et al., 1993) and its rat homologue Ptk-3 (Sanchez et al., 1994). The central portion of the c domain designated as the M AM domain is contained in metalloendopeptidases meprins and the re- ceptor protein tyrosine phosphatase (B eckmann and B ork, 1993).

The multi-function and multi-domain structure of neu- ropilin-1 suggests that each function of neuropilin-1 maps to a distinct region within the extracellular segment of the protein. Previous studies have shown that the class 3 sema- phorins can bind to the a1a2 domains (amino acid [aa] 22–271), a1-b2 domains (aa 22–587), b1-b2 domains (aa 272–587), c domain (aa 588–811), or b1-c domains (aa 272–811) (see Figs. 1 A and 2 A) were constructed as follows. The CDNA fragments upstream and downstream of the domains that would be deleted were amplified by PCR and an EcoRI site added to the 3’-end of the up- stream and 5’-end of the downstream sequences. A flter being checked for PCR errors these sequences were ligated at the EcoRI site and inserted into the eukaryotic expression vector M Iw (Suemori et al., 1990). A s a re- sult of the ligation, the deleted domains were replaced by two amino acids, glutamic acid and phenylalanine, which were translated from the EcoRI sequence GAATTC. To construct NP-abc (see Fig. 1 A), the CDNA en- coding the 1205–1279 aa of X enopus plexin (aa 1230–1237, the transmem-brane domain; see O hta et al., 1995) was amplified by PCR and replaced with the transmembrane-cytoplasmic region of neuropilin-1. The myc tag sequence GGE QK LISEEDL in the NP-abc, NP-abm and NP-am constructs (see Fig. 2 A) was introduced as follows. A N xbal site was added to the 3’-end of the coding region of neuropilin-1 by PCR, and then the Xbal-myc tag-stop codon adapter was ligated. In all mutant neuropilin-1 CDNA s, the signal sequence was retained intact. Construction of the vec- tor for the full-length neuropilin-1 was reported elsewhere (K awakami et al., 1996). To isolate cells that stably express truncated neuropilin-1, L cells, a mouse fibroblastic cell line, were cotransfected with the truncated neu- ropilin-1 CDNAs and pST-neoB (K atoh et al., 1987) according to the calcium phosphate method (Chen and Okayama, 1987) and selected with G ENETICIN (G IBCO-B RL). The sequence data for mouse neuropilin-1 and X enopus plexin are available from G enBank/EM BL/D DB J under the accession numbers D 50086 and D 38175, respectively.

Production of Fc-tagged Recombinant Neuropilin-1 Proteins

CDNAs encoding the full-length of neuropilin-1 extracellular segment (aa 1–852), or the b1-b2 domain-deleted one, were ligated into the expression vector pE F-Fc (M izushima and N agata, 1990; N ishimura et al., 1987) with the adapter of a splicing donor (see Fig. 3 A). The vector was transfected into COS-7 cells using a calcium phosphate precipitation technique. A flter transfection, cells were grown in GIT medium (Wako) for 4 d. The Fc- tagged neuropilin-1 proteins in culture supernatant were purified by M APS-II kit (B io-R ad).

Production of GST-tagged Recombinant Neuropilin-1 Proteins

To produce glutathione-S-transferase (GST)-tagged recombinant proteins, CDNAs for the b1 or b2 domains (see Fig. 3 A) or the deletants of these domains (see Figs. 4 A and 5 A) were amplified by PCR and in- serted into an expression vector, pE X KT-1 (A mershaw Pharmacia Bio- tech). The expression plasmids were transfected to E. coli (strain BL21). The expression of recombinant proteins was induced with 0.1 mM isopro- pyl-β-D-thiogalactoside for 3 h at 37°C. The recombinant proteins, GST- b1 and GST-b2, were recovered as inclusion bodies after sonication of the cells. The inclusion bodies were washed several times with 0.1% PBS con- taining 0.1% (vol/vol) Triton X-100 (PSB) and solubilized with a solu- tion containing 8 M urea, 25 mM 3-cyclohexylamino-1-propanesulfonic acid (CAPS), pH 10.7, and 10 mM EDTA. The denatured proteins were refolded by stepwise dialysis against the following buffers A to E. Buffer A included 1 M urea, 25 mM CAPS, pH 9.8, 2 mM reduced glutathione,  

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1 Abbreviations used in this paper: aa, amino acids; GST, glutathione-S-transferase; VEGF, vascular endothelial growth factor.
0.02 mM oxidized glutathione, 0.005% Tween 80. Buffer B included 1 M urea, 50 mM Tris, pH 9.0, 2 mM reduced glutathione, 0.02 mM oxidized glutathione, 0.005% Tween 80. Buffer C was identical to buffer B but was at pH 8.5. Buffer D replaced the urea of buffer C with 150 mM NaCl. Buffer E contained PBS, pH 8.0. Aliquots tagged b1 and b2 deletionants were soluble, and purified using glutathione Sepharose 4B (Amerham Pharmacia Biotech). The affinity-purified proteins were dialyzed against PBS.

Immunoblot

Proteins were separated by SDS-PAGE (on 10% acrylamide gel in the presence of 2-mercaptoethanol) and transferred onto nitrocellulose membranes as described previously (Towbin et al., 1979). The nitrocellulose membranes were reacted with the rabbit anti-neuropilin-1 antibodies (Kawakami et al., 1996) or anti-myc antibodies (the culture supernatant of hybridoma 9E10; Evan et al., 1985), and then with HRP-conjugated anti-rabbit IgG (Jackson ImmunoResearch) or anti-mouse IgG (Amerham Pharmacia Biotech). Immunoreactivity was detected by the ECL system (Amerham Pharmacia Biotech).

Cell Aggregation Assay

Transfectants and parental L cells were dissociated into single cells with CMF/E and suspended in 10 mM Hapes-buffered CMF (HCMF). To label parental L cells with fluorescent dye, S-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes; 10 μM) was added to the cell suspension. The fluorescein-labeled parental L cells (2.5 × 10^5 cells in 250 μl) and unlabeled transfectants (2.5 × 10^5 cells in 250 μl) were mixed and placed in 24-well culture dishes (SUMILON). To prevent cells from adhering to the wells, the dishes were preincubated with culture medium containing FCS for 1 h. The cell suspensions were agitated at 80 rpm for 30 min or 60 min at 37°C, and then fixed with 4% paraformaldehyde in PBS. The particles were enumerated by hemocytometer. The extent of aggregation was represented by the index Nt/Np, where Nt and Np are the total particle number at incubation times t and 0, respectively (Takeichi, 1977).

Cell Substrate Adhesion Assay

The recombinant neuropilin-1 proteins (65 and 500 μg/ml for the Fc-tagged and GST-tagged recombinant proteins, respectively) were spotted on nitrocellulose-coated culture dishes (Nunc 150288) for 30 min at room temperature as reported (Lemmon et al., 1989; Ohta et al., 1995). The dishes were blocked with 5% skimmed milk in PBS at 4°C overnight, and washed several times with CMF/E containing 100 μg/ml bovine serum albumin (CMFEB). Cells were dissociated with CMF/E and suspended in CMFEB (1 × 10^5 cells/ml). 2 ml of the cell suspension was applied to the dishes and kept at room temperature for 30 min. To test whether semaphorins or VEGF inhibit the neuropilin-1-mediated cell adhesion, the culture dishes with immobilized neuropilin-1 proteins were preincubated with 1.0 μl of affinity-purified myc-tagged chick semaphorin 3A (SEMA3A), or myc-tagged collagen 1A (Kobayashi et al., 1997), a gift from Drs. Raper and Kobayashi) or human VEGF (PEPRO TECH EC C) at room temperature for 30 min, and then added L cell suspension (2 × 10^5 cells in 1 ml) and kept at room temperature for 30 min.

Semaphorin-binding assay

The neuropilin-1 expressing transfectants and parental L cells were incubated with the culture medium containing alkaline phosphatase (AP)-tagged SEMA3A (SEMA3A -AP; gifted from Drs. Raper and Kobayashi) at 37°C for 90 min, washed several times with CMF/E, and then fixed with paraformaldehyde for 1 h. The dishes were washed with PBS and incubated at 65°C for 10 min to inactivate endogenous phosphatase. A flow with 0.1% trypsin lost cell adhesion activity for the neuropilin-1 (NP-full) had cell adhesion activity and made mixed cell aggregates with parental L cells (Fig. 1 C , E, and F) A S L cells whose cell surface proteins had been digested with 0.1% trypsin lost cell adhesion activity for the neuropilin-1-expressing transfectants (data not shown), the cell adhesion ligand(s) for neuropilin-1 on L cells appears to be a protein. The strength of cell adhesion shown by the transfectants expressing the a1-a2 domain deleted neuropilin-1 (NP-bc) was similar to that of the transfectants with full-

Plexin Binding Assay

The expression vectors for the mouse plexinA1 subfamily members (plexinA1, plexinA2, and plexinA3; Kameda et al., 1996a) were constructed as follows. The plexin cDNAs whose native signal sequences were replaced to the one of the Sema3A (aa 1–25) and myc-tag sequences were inserted into the expression vector, pCA AGG S (Niwa et al., 1991). The expression vector was transfected into the COS-7 cells by Lipo- FECTAMINE (GIBCO-BRL) following the manufacturer’s protocol. After the mixture of recombinant proteins (GST-b1 [347–364] and GST-b2 [504–521]) were added to the culture medium, and incubated at 37°C for 1 h. After washing with the culture medium, the cells were fixed with paraformaldehyde for 1.5 h at room temperature. A filter with the rinsing with PBS(T), the dishes were treated with 0.1% H2O2 in PBS(T) to inactivate the endogenous peroxidase. The recombinant proteins bound to the cell surfaces were detected by the combination of the anti-GST monoclonal antibody (CLONTECH), HRP-conjugated secondary antibody, and DAB. The sequence data for the mouse plexinA1, plexinA2, and plexinA3 are available from GenBank/EMBL/DDBJ under the accession numbers D86948, D86949, and D86950, respectively.

Image Acquisition and Analysis

Fluorescence images were obtained using a charged-coupled device (CCD) camera (Photometrics), digitized, and processed by background subtraction and contrast enhancement using IPLab Spectrum (Scalinics). Light transmission or phase contrast microscopic images were obtained using a HC-2500 CCD camera system (FUJIFILM) and digitized by the photonlab-2500 software (FUJIFILM).

Results

Identification of Cell Adhesion Domains of Neuropilin-1

To identify the domains of the neuropilin-1 protein involved in cell adhesion, we first analyzed the cell adhesion activity of the transfectants which express mutant neuropilin-1 lacking the a1-a2 domains (Fig. 1 A; NP-bc) or the a1-b2 domains (Fig. 1 A; NP-c), and the protein in which the transmembrane and cytoplasmic domains were replaced with the transmembrane domain and NH2-terminal 22 amino acids of the cytoplasmic domain of Xenopus plexin (Fig. 1 A; NP-abcp). We isolated several transfectants that expressed different amounts of mutant neuropilin-1 proteins (Fig. 1 B). A II of the transfectants were immunoreactive with an antibody generated against the b2-c domains of mouse neuropilin-1 (Kawakami et al., 1996) in living condition (data not shown), indicating that the mutant proteins were expressed on cell surfaces.

The cell adhesion activity of the mutant neuropilin-1 proteins was determined by cell aggregation assay; a 1 to 1 mixture of transfectants and fluorescein-labeled parental L cells was agitated at 80 rpm in CMF, and then particles were enumerated at 30 or 60 min. Parental L cells did not form aggregates (Fig. 1, C and D), indicating that L cells do not show cell adhesiveness in this conditions. In contrast, transfectants expressing intact neuropilin-1 protein (NP-full) had cell adhesion activity and made mixed cell aggregates with parental L cells (Fig. 1, C, E, and F). A S L cells whose cell surface proteins had been digested with 0.1% trypsin lost cell adhesion activity for the neuropilin-1-expressing transfectants (data not shown), the cell adhesion ligand(s) for neuropilin-1 on L cells appears to be a protein. The strength of cell adhesion shown by the transfectants expressing the a1-a2 domain deleted neuropilin-1 (NP-bc) was similar to that of the transfectants with full-

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length neuropilin-1 (Fig. 1 C). Mutant neuropilin-1 in which the transmembrane and cytoplasmic region had been replaced by the corresponding regions of Xenopus plexin (NP-abcp) also showed cell adhesion activity in proportion to the expression level of the mutated proteins (compare Fig. 1, B with C). In contrast, the transfectants expressing the c domain but not a1-b2 domains (NP-c) showed no cell adhesion activity (Fig. 1 C), even though sufficient amounts of truncated proteins were expressed (Fig. 1 B). These results suggest that the b1-b2 domains but not a1-a2 or transmembrane-cytoplasmic domains are involved in the heterophilic cell adhesion.

To confirm further the involvement of the b1-b2 domains in cell adhesion, we produced other lines of transfectant in which the b1-b2, c, or b1-c domain were deleted (Fig. 2 A). As the anti–neuropilin-1 antibody was generated against the b2-c domains (Kawakami et al., 1996), the antibody was inadequate to detect mutant neuropilin-1s lacking these domains. Therefore, we introduced myc tag into the COOH-terminal end of the mutant neuropilin-1 proteins. Each transfectant expressed mutant neuropilin-1 proteins of expected molecular size (Fig. 2 B and C). Cell aggregation analysis demonstrated that the transfectants expressing the b1-b2 domain-deleted neuropilin-1 (NP-ac) or the b1-c domain deleted neuropilin-1 (NP-am) did not show cell adhesion activity (Fig. 2 D). On the other hand, the transfectants expressing the c domain-deleted neuropilin-1 (NP-abm) showed cell adhesion activities, proportional to the expression levels of the mutant proteins (Fig. 2, B, C, and D).

Collectively, the results obtained in cell aggregation analysis indicate that the b1-b2 domains play an important role in neuropilin-1–mediated heterophilic cell adhesion, and suggest that the cell binding site(s) is located within the domains.

**Cell Adhesion Activity of Recombinant Neuropilin-1 Proteins**

To determine whether the b1 and b2 domains possess cell adhesion activity, we produced Fc-tagged recombinant proteins for the full-length of neuropilin-1 ectodomain (NP-Fc) and the b1-b2 domains–deleted one [NP(b-)-Fc] by COS-7 cells and GST-tagged recombinant proteins for the b1 (GST-b1) and b2 (GST-b2) domains in E. coli, and tested their cell adhesion activity by cell substrate adhesion assay. Each recombinant protein was absorbed on nitrocellulose-coated culture dishes, and then L cells were applied to the dishes. As shown in Fig. 3 C, L cells adhered to the NP-Fc-, but not NP(b-)-Fc, absorbed culture dishes. L cells also adhered to the immobilized GST-b1 or GST-b2 but not GST; Fig. 3 C used as a control substrate. These results were well coincided with the results obtained by the cell aggregation assay described above, and further suggest that neuropilin-1 has at least two cell adhesion sites, one in the b1 domain and the other in the b2 domain.

**Determination of Cell Adhesion Sites within the b Domain**

To identify cell adhesion sites within the b domain, a series of peptides for these domains were synthesized, and tested their cell adhesion activity by cell substrate adhesion assay.

The hydrophathy profile of the b2 domain indicated that the central region around a putative glycosylation site (aa
501–547) was most hydrophilic (Fig. 4 A). Therefore, we thought that cell adhesion sites might exist within this region, and tested cell adhesion activity of the GST-tagged recombinant protein for this region. As expected, the GST-tagged peptide for aa 501–547 within the b2 domain (GST-b2[501-547]) showed cell adhesion activity (Fig. 4 C), indicating that a cell adhesion motif of the b2 domain is located within this region. We next produced GST-tagged peptides in which the NH₂ and COOH termini of this region were stepwise deleted (Fig. 4, A and B) and tested their cell adhesion activity. As shown in Fig. 4, D–H, 18 amino acid residues (aa 504–521; GST-b2[504–521]) were sufficient to mediate cell adhesion. Trypsin-treated L cells did not adhere to GST-b2[504–521] (Fig. 4 I), indicating that the cell adhesion ligand(s) recognized by the peptide is a protein.

On the assumption that the cell adhesion site within the b1 domain exists in the region homologous to the cell adhesion site within the b2 domain, we prepared a GST-tagged peptide for aa 347–534 of the b1 domain (Fig. 5 A and Fig. 4 B, lane 8), and tested its cell adhesion activity. As shown in Fig. 5 B, L cells adhered to the immobilized...
GST-tagged peptide, GST-b1(347–364). Trypsin-treated L cells did not adhere to the recombinant protein (Fig. 5 C). These results indicate that the cell adhesion activity in the b1 domain also exists in the homologous 18-amino acid sequences in the b2 domain.

**Adhesion of Embryonic Cells and Line Cells to Recombinant Neuropilin-1 Proteins**

To examine which types of cell adhere to neuropilin-1, we performed cell substrate adhesion assay for primary embryonic cells and line cells derived from different origins, including HEK 293T (human embryonic kidney), COS-7 (monkey kidney), Hela (human cervix carcinoma), p19 (mouse embryonic carcinoma), K B (human epidermoid carcinoma), and NIH3T3 (mouse embryo).

Trunk mesenchymal cells from E13 mouse embryos that had been dissociated from the primary culture with EDTA adhered to the GST-b1(347–364) and GST-b2(504–521) recombinant proteins (Fig. 6, A and C). In contrast, when the cells were dissociated with trypsin, they did not adhere to the substrates (Fig. 6, B and D). These results suggest that a cell adhesion ligand(s) for neuropilin-1 exists on the surfaces of embryonic cells and is a protein. All of the line cells examined adhered to the GST-b1(347–364) and GST-b2(504–521) recombinant proteins (data not shown), suggesting that the cell adhesion ligand(s) for neuropilin-1 is a ubiquitous membrane protein.

**Interaction of Semaphorins, VEGF, and Plexins to the Cell Adhesion Sites of Neuropilin-1**

Neuropilin-1 is shown to interact with the class 3 semaphorins (Chen et al., 1997; Feiner et al., 1997; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997), VEGF165 (Soker et al., 1998), and the plexinA subfamily members (Takahashi et al., 1999; Tamagnone et al., 1999). Therefore, we tested whether these molecules can interact with the cell adhesion sites of neuropilin-1 and mediate cell adhesion.

First, we tested the binding of SEMA3A-AP to the transfectants expressing mutant neuropilin-1s. The transfectants were incubated with the culture supernatant containing SEMA3A-AP (20 collapse units). Strong AP staining was observed in the transfectants expressing NP-full (Fig. 7 A), NP-ac (Fig. 7 C), and NP-abm (Fig. 7 D). In contrast, SEMA3A-AP bound very weakly to the transfectants expressing NP-bc (Fig. 7 B) or NP-am (Fig. 7 E), even though sufficient amounts of the truncated neuropilin-1 proteins were expressed in these transfectants (see Figs. 1 B and 2 C). No SEMA3A-AP binding was observed for the cells expressing NP-c (Fig. 7 F) or parental L cells (data not shown). These results indicate that semaphorin 3A can bind to the b1 and b2 domains which contain the cell adhesion sites of neuropilin-1, as well as the a1-a2 domains.

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adhesion sites of neuropilin-1. These results suggest that the class 3 semaphorins do not interact with the cell adhesion sites of neuropilin-1. This was further confirmed by the competition assay. The presence of SEMA3A (6.7 nM) did not inhibit the adhesion of L cell to NP-Fc (Fig. 8 B). In addition, we also showed that VEGF165 (100 nM) did not interfere with the cell adhesion activity of neuropilin-1 (Fig. 8 C).

Finally, we examined whether the cell adhesion sites of neuropilin-1 interact with the plexinA subfamily members. We expressed the mouse plexinA1, plexinA2, and plexinA3 in COS-7 cells, applied the mixture of GST-b1(347–364) and GST-b2(504–521) recombinant proteins (45 μg/ml each), and monitored the binding of the recombinant proteins by anti-GST antibody. We did not observe prominent binding of GST-b1(347–364) and GST-b2(504–521) to the plexinA3-expressing cells (Fig. 8, D and E), or to plexinA1- and plexinA2-expressing cells (data not shown).

Collectively, these results suggest that the class 3 sema-
phorins, VEGF, or the plexinA subfamily members do not interact with the cell adhesion sites of neuropilin-1, and are not cell adhesion ligands for neuropilin-1.

**Discussion**

Neuropilin-1 is a molecule with multi-domains and multi-functions. The present structure-function analyses on neuropilin-1 identified the sites involved in cell adhesion.

Our previous study had indicated that L cells acquire cell adhesiveness when they are transfected with neuropilin-1 cDNA and express the proteins on their surface. However, it was not known whether the cytoplasmic segment of neuropilin-1 is required for the cell adhesion activity as it is in cadherins (Nagafuchi and Takeichi, 1988). In this study, we constructed a mutant neuropilin-1 cDNA in which the transmembrane and cytoplasmic regions had been replaced by the corresponding regions of *Xenopus* plexin, transfected L cells, and isolated cell lines expressing the mutant neuropilin-1 protein, NP-abcp. Cell aggregation analysis showed that the NP-abcp-expressing cells adhered as well as the cells expressing the full-length of neuropilin-1. Plexin is a cell adhesion molecule but its cytoplasmic segment is not required for the cell adhesion activity (Ohta et al., 1995). In addition, this study showed that the Fc-tagged recombinant protein for the extracellular segment of neuropilin-1 retained cell adhesion activity. Therefore, we can conclude that the extracellular part of neuropilin-1 is sufficient to mediate cell adhesion.

The present cell aggregation analyses using L cells in which the domains in the extracellular segment of neuropilin-1 had been deleted showed that the b1/b2, but not a1/a2 or c, domains were essential to the cell adhesion activity of neuropilin-1. As L cells adhered to the recombinant proteins for the b1 and b2 domains, but not the b1-b2 domains-deleted protein, these two domains can mediate cell adhesion independently. Furthermore, cell substrate adhesion assays for a series of recombinant proteins for the b1 and b2 domains clarified that there are two cell adhesion sites with a stretch of 18 amino acid residues in the central part of these domains.

A search of the databases shows that the cell adhesion sites within the b1 and b2 domains are highly conserved among *Xenopus* (Takagi et al., 1991), chicken (Takagi et al., 1995), and mouse neuropilin-1s (Takagi et al., 1995). However, it was not known whether the cytoplasmic segment of neuropilin-1 is required for the cell adhesion activity as it is in cadherins (Nagafuchi and Takeichi, 1988).

![Figure 8](image_url)

Figure 8. Binding of semaphorins, VEGF, and plexins to the recombinant neuropilin-1 proteins. (A) Bindings of SEMA3A-AP, Sema3B-AP, and Sema3C-AP to the immobilized NP-Fc, GST, GST-b1, GST-b2, GST-b1(347–364), and GST-b2(504–521) were visualized with NBT/BCIP. (B and C) Adhesion of L cells to the immobilized NP-Fc in the presence of SEMA3A and VEGF (D). SEMA3A and VEGF do not interfere with the binding of L cells to NP-Fc. (D and E) Binding of GST-tagged recombinant proteins for the cell adhesion sites of neuropilin-1 to myc-tagged plexinA3 expressed in COS-7 cells. The plexinA3 was visualized by immunohistochemistry with anti-myc antibody (D). GST-b1(347–364) and GST-b2(504–521) bound to the cells were detected by immunohistochemistry with anti-GST antibody. Bars, 100 μm.

![Figure 9](image_url)

Figure 9. Comparison of amino acid sequences in cell adhesion sites among vertebrate neuropilin-1s (A) or the mouse neuropilin family (B). The amino acid sequences in the cell adhesion sites are highly conserved among the mouse (mNP), rat (rNP), human (hNP), chicken (cNP), and *Xenopus* (xNP) neuropilin-1s. On the other hand, only about half of the amino acid residues of the cell adhesion site of mouse neuropilin-1 and of mouse neuropilin-2 are identical.
Doms similar to the b1/b2 of neuropilin-1 are shared by various molecules, including coagulation factors V and VIII (Toole et al., 1984; Jenny et al., 1987), DDR/Ptk-3 (Johnson et al., 1993; Sanchez et al., 1994), and MFGP (Stubbs et al., 1990; Larocca et al., 1991). However, the amino acid sequences in the cell adhesion sites of neuropilin-1 are unique. Furthermore, the cell adhesion sites with an 18-amino acid stretch do not contain any motifs reported so far, suggesting that neuropilin-1 is an unique cell adhesion receptor. Interestingly, the cell adhesion sites in the b1 and b2 domains show little homology (see Fig. 5A). It is likely that each site can interact with distinct cell adhesion ligands or distinct regions of the same ligand.

Several studies have reported that neuropilin-1 can bind to the class 3 semaphorins (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997), and human (He and Tessier-Lavigne, 1997; K o lodkin et al., 1997) neuropilin-1s (Fig. 9A), suggesting that cell adhesion activity is a universal function of neuropilin-1. A mother member of the neuropilin family, neuropilin-2, also possesses a similar domain structure to neuropilin-1 (Chen et al., 1997). However, the amino acid sequences of the cell adhesion sites of neuropilin-1 do not closely resemble the corresponding regions of neuropilin-2 (Fig. 9B). Therefore, it is an open question whether neuropilin-2 can mediate cell adhesion as neuropilin-1 does.

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