Transmembrane Domain Interactions Control Biological Functions of Neuropilin-1

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Neuropilin-1 (NRP1) is a transmembrane receptor playing a pivotal role in the control of semaphorins and VEGF signaling pathways. The exact mechanism controlling semaphorin receptor complex formation is unknown. A structural analysis and modeling of NRP1 revealed a putative dimerization GxxxG motif potentially important for NRP1 dimerization and oligomerization. Our data show that this motif mediates the dimerization of the transmembrane domain of NRP1 as demonstrated by a dimerization assay (ToxLuc assay) performed in natural membrane and FRET analysis. A synthetic peptide derived from the transmembrane segment of NRP1 abolished the inhibitory effect of Sema3A. This effect depends on the capacity of the peptide to interfere with NRP1 dimerization and the formation of oligomeric complexes. Mutation of the GxxxG dimerization motif in the transmembrane domain of NRP1 confirmed its biological importance for Sema3A signaling. Overall, our results shed light on an essential step required for semaphorin signaling and provide novel evidence for the crucial role of transmembrane domain of bitopic protein containing GxxxG motif in the formation of receptor complexes that are a prerequisite for cell signaling.

INTRODUCTION

Many cellular signaling pathways require the formation of homo- and hetero-oligomers of proteins in order to ensure appropriate signal integration. This concept emerged from the discovery that most protein components of these pathways contain discrete and modular protein–protein interaction domains (Sudol, 1998; Pawson, 2004). These domains have so far been characterized mainly for soluble cytoplasmic proteins. However, the assembly of membrane proteins into receptor complexes is also of prime importance for cellular signaling. Examples include the activation of tyrosine kinase receptors through dimerization (Weiss and Schlessinger, 1998; Schlessinger, 2002), GPCR dimerization (Breitwieser, 2004), the multimeric nature of cytokine receptors and a multitude of other receptors such as T-cell receptor, semaphorin receptors, and integrins (Giancotti and Ruoslahti, 1999; Davis *et al.*, 2003; de Caestecker, 2004). Many of the proteins shown to be involved in the formation of such signaling receptor complexes are single transmembrane (TM) domain proteins (bitopic proteins). Homo- or heterodimerization is often crucial for signal transduction, but the nature of the mechanisms controlling the formation of the complexes, the mode of selective recruitment of the coreceptors and the dynamics of these processes remain open questions. Theoretical considerations of the folding of alpha-helical TM segments led to the proposal that interactions

between TM helices may play a significant role in receptor complex assembly and TM signaling (Bormann and Engelman, 1992). This active role depends on the propensity of helices to associate and thus upon the existence of specific dimerization motifs in their sequence. Characterization of these motifs has led to significant progress over the past few years. Currently, the human erythrocyte glycoprotein A (GPA) is the most characterized protein presenting TM domains interactions. Its dimerization is due to strong interactions between the TM domains of two monomers. Mutagenesis studies have defined the amino acid motif involved in the interactions (Lemmon *et al.*, 1992), and a reporter gene dimerization assay (TOXCAT) has confirmed this motif (Russ and Engelman, 1999). The interaction has also been quantified by FRET (Fisher *et al.*, 1999). Final confirmation of these data are provided by the elucidation of the NMR structure of the GPA TM domain dimer (MacKenzie *et al.*, 1997). The core of the dimerization motif is a GxxxG sequence (where x represents any amino acid; Senes *et al.*, 2000) and relates to glycine interactions (Smith *et al.*, 2001). Such a motif has been shown to be important for the dimerization of TM segments of ErbB receptors (Mendrola *et al.*, 2002). Consistently with previous work by Marchesi and colleagues (Bormann *et al.*, 1989), we have also recently demonstrated that synthetic peptides mimicking TM domains are able to inhibit specifically kinase activity and cell signaling by EGF (erbB1) and erbB2 receptors in cultured cells (Bennasroune *et al.*, 2004) as well as chimeric insulin receptors containing these domains (Bennasroune *et al.*, 2005). This inhibition indeed relates to the presence of the GxxxG-type sequence. These results led us to examine the TM of neuropilin-1 (NRP1), the ligand binding subunit of the semaphorin receptor complex in particular for Sema3A, the founder member of the semaphorin family (Kolodkin *et*

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al., 1993, 1997). The semaphorins are one of the three major families of diffusible or membrane-bound signals (together with the netrins and ephrins) that perform critical functions during embryonic brain development (Tessier-Lavigne and Goodman, 1996). The semaphorins are a family of more than 25 members subdivided into eight classes according to their structural specificities. In addition to axon guidance, semaphorins also regulate cell migration, cell differentiation, and apoptosis in both physiological and pathological conditions (Pasterkamp and Verhaagen, 2001; Fiore and Puschel, 2003; Hinck, 2004; Kruger *et al.*, 2005). This functional diversity is due to the formation of a highly dynamic receptor complex by the selective recruitment and activation of multiple intracellular pathways, leading to actin cytoskeleton remodeling (Castellani and Rougon, 2002). Interestingly, NRP1 possesses a short intracellular domain without clear transduction capacity. NRP1 is therefore recruiting various partners such as members of the plexin family (Tamagnone *et al.*, 1999), other adhesion molecules (L1, NrCAM; Castellani *et al.*, 2000; Falk *et al.*, 2005), tyrosine kinase receptors (VEGFR1, VEGFR2, MET; Soker *et al.*, 1998; Bagnard *et al.*, 2001b; Winberg *et al.*, 2001), or integrins (Pasterkamp *et al.*, 2003). How the receptor complexes are forming remains elusive. Moreover, because of the pivotal physiological role of NRP1 in a variety of normal (organogenesis, angiogenesis, CNS development, and maturation) and pathological conditions (CNS lesions, cancer progression), we decided to analyze the molecular mechanism initiating receptor complex formation in response to semaphorins. In particular, we focused on the NRP1-dependent Sema3A signaling because it is probably the best documented (Castellani and Rougon, 2002). Here, we report that the TM domain of NRP1, which contains a double GxxxG motif, suggesting an important role for this domain in receptor dimerization, is important for signal transduction. This is supported by structural modeling of the TM domain of NRP1 and biochemical and functional assays demonstrating that the TM domain drives dimerization of NRP1 via its GxxxGxxxG motif. Thus, we show that this motif is important for the formation of the oligomeric receptor complex and is required to trigger semaphorin signaling.

EXPERIMENTAL PROCEDURES

Peptide Preparation

G. Nullans and B. Guérard synthesized peptides within the U575 proteomic facility using Fmoc chemistry automatic peptidic synthesis (A33A; Applied Biosystems, Foster City, CA) except for the GPA peptide, which was purchased from Neosystem (Strasbourg, France). Peptides masses were analyzed by matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (Bruker Biflex, Wissembourg, France). The original C-terminal juxtamembrane sequence of the NRP1 TM peptide (CTC) was replaced by a more classical tribasic sequence (RKR) as found in many membrane proteins in order to reduce possible artifactual formation of disulfide bridges. We have previously shown that other TM peptides containing such a tribasic C-terminus do readily incorporate into the plasma membrane of cultured cells (Bennasroune *et al.*, 2004, 2005). The first peptide corresponding to the TM sequence of NRP1: ILITIIAMSALGVLLGAVCGVLYRKR (in one-letter code, amino acids ¹⁸⁵⁷ to ^{Y880} plus RKR according to Swissprot entry P97333) will be referred to as pTM-NRP1. In the second peptide, the glycine residues have been replaced by valine residues (ILITIIAMSALVLLVAVCVVLYRKR). This peptide will be referred to as mutated TM peptide, or pTM-NRP1^{mut}. Peptide purity estimated by reverse phase-HPLC (RP-HPLC, Beckman Gold apparatus, Vydac 219-TP510 diphenyl columns) was higher than 90%. Peptides were routinely prepared in a micellar form in 7 mM LDS (lithium dodecylsulfate), and administered to cells in a small volume so that detergent concentration fell well below its critical micelle concentration (CMC), ensuring partitioning of the peptides in cell membranes (Bennasroune *et al.*, 2004).

Labeling and HPLC Purification of the TM Peptides

For all biological experiments, the peptides were de-protected and purified by reverse-phase HPLC (Beckman Gold apparatus, Fullerton, CA; Vydac 219-TP510 diphenyl columns, Hesperia, CA). For the FRET experiments, protected and resin-bound peptides were chemically labeled with 1-pyrene butanoic acid (Molecular Probes, Eugene, OR) or dimethyl-amino-coumarin-acetic acid succinimidyl ester (DAMCA-SE; Fluoroprobes, Interchim, Montluçon, France) in dimethylsulfoxide (Fisher *et al.*, 1999). After de-protection, peptides were purified by RP-HPLC (Beckman Gold apparatus, Nucleosil C4 or Phenyl Macherey-Nagel (Hoerd, France) semi-preparative columns. Fluorescent peptide masses were checked by matrix-assisted laser-desorption/ionization time-of-flight mass spectroscopy on a Voyager DE-RP (Applied Biosystems, Foster City, CA).

Modeling of the Neuropilin-1 TM Peptide Dimer

A conceivable model of a dimeric form for the TM domain of neuropilin-1 was built based on the structure of the right-handed GPA dimer (PDB code 1AFO; MacKenzie *et al.*, 1997) using the Swiss-PDB viewer program (Guex and Peitsch, 1997). This model was made by aligning the first GxxxGxxxG motif of NRP1 TM sequence with the well-characterized motif of GPA, GxxxGxxxT. Manual adjustment of the resulting structure was performed in order to obtain better packing of the two monomers. The energy of the resulting structure was minimized in vacuo, excluding electrostatic interactions by using Swiss-PDB viewer.

Estimation of TM-NRP1 Dimerization (ToxLuc Method)

TM domains homodimerization capacity was estimated using a modified TOXCAT system (Russ and Engelman, 1999). This system measures TM helices association in *Escherichia coli* internal membrane. The method is based on the oligomerization of the transcription activator ToxR, which occurs only through TM domain dimerization. The TM sequences of interest were placed in a fusion protein between the ToxR element (intracellular) and the extracellular maltose-binding protein (MBP). When TM domain interactions induced dimerization of this fusion protein, the reporter gene was activated. Here, we used a modified version in which the reporter gene encodes for the luciferase protein (Bennasroune *et al.*, 2005). TM domains of NRP1, mutated NRP1, ErbB2, GPA, and mutated GPA were used for fusion protein construction. Luciferase luminescence was measured using a kit from Roche (Indianapolis, IN) and a Berthold Microplate luminometer (Pforzheim, Germany). Western blot analysis of MBP protein content confirmed equal production of the different constructions.

Fluorescence Resonance Energy Transfer Measurements

Reconstitution in detergent micelles and fluorescence resonance energy transfer (FRET) measurements were performed as previously described by Fisher *et al.* (1999, 2003). Briefly, an equimolar mixture of pyrene- and coumarin-labeled peptides was adjusted to 5–20 μ M total peptides in trifluoroethanol (TFE) and was mixed with a small volume of 1 M lauryldimethylamine-oxide (LDAO; Fluka, L'Isle d'Abeau, France). This solution was dried in a SpeedVac and resuspended in PBS (20 mM phosphate buffer, pH 7, 137 mM NaCl), supplemented with 5 mM dithiothreitol (DTT), in order to obtain a final detergent concentration of 5 mM. Fluorescence measurements were performed using a Jobin Yvon Fluorolog FL3-21 spectrofluorimeter (Edison, NJ). Fluorescence excitation spectra were recorded, and the relative contribution of pyrene (sensitized emission, with its characteristic peak at 345 nm) and coumarin (direct emission, with its characteristic peak at 370 nm) to the fluorescence emission at 500 nm was calculated. This FRET ratio provides a measure of the degree of dimerization of the two peptides (Fisher *et al.*, 1999). An apparent dissociation constant was calculated from the dependence of the FRET signal on the peptide concentration, whereas the detergent concentration remained constant. Details of the equations used to perform the calculations are given in Duneau *et al.* (2007).

Functional Study on Cortical Explants

Cortical explants prepared from E15 mouse embryos were grown on glass coverslips coated with Laminin (1 mg/ml)/poly-L-lysine (10 mg/ml; all from Sigma) as previously described (Bagnard *et al.*, 2000). Explants were kept at 37°C under 5% CO₂ in air. A sufficient radial outgrowth could be seen after 48 h in culture and individual fibers and growth cones could then be analyzed. Sema3A (100 ng/ml) or combination of Sema3A (100 ng/ml) with pTM-NRP1 (at concentrations of 10⁻⁸, 10⁻⁹, 10⁻¹⁰, and 10⁻¹² M in LDS or 10⁻⁸ M in DMSO), pTM-NRP1^{mut} (10⁻⁸ M), or pTM-ErbB2 (10⁻⁸ M) were added for 2 h in the culture medium. Cultures were fixed in 4% formaldehyde. For each explant, the total number of growth cones and collapsed growth cones was determined (Bagnard *et al.*, 2001a). Experiments were analyzed in blind conditions by two independent analysts in some cases.

Plasmids and Transfection

Plasmids were provided by Pr AW. Püschel (Münster Universität, Germany). NRP1 and PlexinA1 (PlexA1) cDNAs have been cloned in pBK-CMV (Strat-

agene, La Jolla, CA; Rohm *et al.*, 2000). A plasmid encoding for a NRP1 protein with the triple (G→V) mutation in the TM region was generated with the help of MilleGen (Toulouse, France). This plasmid was checked by sequencing. Routinely, 80% confluent COS cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions in six-well plates. Two micrograms of each plasmid was added. Experiments were performed 48 h after transfection.

Functional Study on COS Cells

COS-1 cells (ATCC, Manassas, VA; CRL-1650) were grown in DMEM medium with 4.5 g glucose/l (GIBCO, Rockville, MD), 5% fetal bovine serum, 580 mg/l glutamine, and antibiotics. NRP1- and PlexA1-transfected COS cells (see details in *Plasmids and Transfection*) were cultured on 12-well plates with previously poly-L-lysine-coated glass coverslips (0.005 mg/ml). The COS cells were incubated 1 h with 1 nM pTM-NRP1 or 1 nM pTM-NRP1^{mut} at 37°C before treatments. Culture medium was removed and replaced by conditioned medium obtained from HEK293 cells stably expressing Sema3A or mock-transfected cells (Sema3A and control medium; see Bagnard *et al.*, 1998 for details) for 4 h at 37°C. Cells were fixed in 4% formaldehyde for 15 min. In certain experiments, we coexpressed PlexA1 with the mutated form of NRP1 instead of the wild-type NRP1. For each condition tested, 400 cells were analyzed in three independent experiments. Statistical analysis was performed by using χ^2 analysis.

Binding Assay

Wild-type COS-1 cells, NRP1-expressing COS-1 cells (COS-NRP1), or NRP1^{mut}-expressing COS-1 cells (COS-NRP1^{mut}) were cultured on 96-well plates (Perkin Elmer-Cetus Life Science, Boston, MA) previously coated with poly-L-lysine (0.005 mg/ml). Cells were washed with serum-free medium and incubated with TM peptide freshly diluted in serum-free medium for 1 h at 37°C. After 90-min saturation with serum at 37°C, the culture medium was replaced by conditioned medium containing alkaline phosphatase-coupled Sema3A (AP-Sema3A) obtained from AP-Sema3A stably expressing HEK cells for 2 h at 4°C (Bagnard *et al.*, 1998). Cells were washed with PBS and fixed in 4% formaldehyde for 15 min. After three washes in PBS, the plate was warmed for 50 min at 65°C. Cells were subsequently incubated with 80 μ l of AP luminescent substrate (Lumi-phos, Lumigen, Southfield, MI) to determine luminescence after 15 min with Microumat Plus system (Berthold Technologies). To analyze vascular endothelial growth factor (VEGF) binding, culture medium was removed 2 d after transfection and replaced by VEGF-containing (R&D Systems, Minneapolis, MN) serum-free medium (50 ng/ml). After 1-h incubation at 20°C, cells were washed and harvested with a boiling buffer (Tris 50 mM/EDTA 5 mM, pH 6.8) containing 0.1 M DTT, and 10% SDS. Bound VEGF and NRP1 or NRP1^{mut} content were analyzed by Western blotting, using anti-VEGF (R&D Systems) at a dilution of 1:250 or polyclonal anti-VSV (Sigma) at a dilution of 1:20,000. Immunoreactivity was detected with an enhanced chemiluminescence blot detection system in accordance with the manufacturer's instructions (Supersignal, Pierce, Rockford, IL), registered, and analyzed thanks to a computerized GeneGnome imager (Syngene, Cambridge, United Kingdom). Statistical analysis was performed by using Student's *t* test.

Sedimentation Constant Analysis by Centrifugation in Sucrose Gradients

Confluent COS-1 cells expressing NRP1 and PlexA1 were washed and incubated with TM peptides freshly diluted in serum-free medium for 1 h at 37°C. The culture medium was replaced by conditioned medium containing AP-Sema3A for 1 h at 37°C (Bagnard *et al.*, 1998). Cells were harvested with 10 mM EDTA and centrifuged. The pellet was washed in PBS and then diluted in lysis buffer (Tris-HCl/NaCl, 50/150, pH 8.0) composed of 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate (DOC), 2 mM vanadate, and protease inhibitors (Pierce) for 90 min at 4°C. Sucrose density gradient sedimentation experiments were based on step gradients containing 25, 17, 10, and 3% sucrose. Solutions were made in HEPES/NaCl buffer (30/30, pH 7.6, 0.12% Triton; Leray *et al.*, 1992). Cell lysates (40 μ l) were placed on the top of the gradient in an ultracentrifuge tube and centrifuged at 100,000 $\times$ g for 105 min with a TL-100 ultracentrifuge (Beckman; rotor TLS-55). Fractions were collected from the bottom and subjected to SDS-PAGE at constant voltage and temperature in a buffer 0.025 M Tris, 0.192 M glycine, pH 8.3, and 0.01% SDS. Proteins were transferred to nitrocellulose membrane. Electrophoretic transfer occurred at 4°C for 3 h in a buffer 20% ethanol, 0.025 M Tris, 0.192 M glycine, pH 8.3, and 0.01% SDS. The membrane was blocked for 1 h with PBS/BSA 5% and then incubated overnight with polyclonal anti-VSV (vesicular stomatitis virus; Sigma) at a dilution of 1:20,000. The membrane was washed three times in PBS/0.1% Tween 20 before incubation with the secondary antibody (anti-mouse, 1:1000) for 1 h. Immunoreactivity was detected with an enhanced chemiluminescence blot detection system in accordance with the manufacturer's instructions (Supersignal, Pierce). The whole experiment was performed at room temperature. For sedimentation constant(s) evaluation, the gradients were calibrated with thyroglobulin (19.3S), ferritin (17.7S), catalase (11S), lactate dehydrogenase (7S), and albumin (4.3S; high-molecular-weight

calibration kit for electrophoresis, Pharmacia Biotech, Piscataway, NJ) as previously described (Fritsch, 1973). We predicted the assembled state of NRP1 from sedimentation constants using Kirkwood-Riseman theory (Bloomfield *et al.*, 1967). We considered that NRP1 contains seven autonomous domain including TM and intracellular domains. We hypothesized an elongated multidomain protein for NRP1, compatible with an ellipsoid with an axial ratio of $\sim$ 8. This hypothesis is in agreement with the low resolution structure of procollagen C-proteinase enhancer (PCPE), another protein with multidomain (two CUB domain and a Netrin-like domain) demonstrated to be an elongated multidomain glycoprotein (Bernocco *et al.*, 2003). Thus, we can predict a sedimentation constant of 6S for a monomer and 9S for a side-by-side dimer of NRP1.

RESULTS

Predicted Structural Features and Modeling of the NRP1 TM Peptide

During search for specific sequence mediating semaphorin receptor dimerization we recognized a double GxxxG motif in the TM domain of NRP1. This observation suggested that dimerization of NRP1 could occur through this motif known to promote higher order structures in TM helical proteins (Russ and Engelman, 2000). The structure of this dimer illustrates how aligned glycines on one face of the helix form a groove that allows for close packing of the two helices. As illustrated in Figure 1, the NRP1 TM sequence contains two contiguous such motifs. Helical wheel representation of this sequence shows that the three glycines are indeed located on a same face of the helix (Figure 1A). This is supported by a molecular representation of the NRP1 TM domain monomer (Figure 1C). We also built a possible model of the NRP1 TM dimer, based on the right-handed structure of GPA (MacKenzie *et al.*, 1997). The model shows a very good packing of the two monomers, without any clash of amino acid side chains. A view along the dimer interface highlights the three glycines (in red), which form a flat groove into which adjacent residues can pack (Figure 1D). A view down the dimer axis also shows the close packing of the two helices allowed by the alignment of the glycine residues (Figure 1B).

TM NRP1 Has a Strong Dimerization Capacity

The ToxLuc system (Bennasroune *et al.*, 2005) is a modification of the TOXCAT system (Russ and Engelman, 1999) originally designed to screen for TM domains that mediate dimerization; we therefore used this system to assess TM NRP1 dimerization. Different vectors encoding the TM sequences known to mediate dimerization were used as references for dimerization capacity. The ToxLuc system revealed a strong dimerization capacity of TM NRP1, significantly higher than that of ErbB2 ($\times$ 5.3) and slightly superior to that of Glycophorin A ($\times$ 1.4; Figure 2A). The amino acid sequence of the TM NRP1 favored dimerization of the TM domain of NRP1, thus validating results of the modelization described above. Furthermore, the ToxLuc system revealed no binding capacity of the TM-NRP1^{mut} in which the three glycines of the GxxxG motif are replaced by valines. We also performed a FRET analysis of the TM NRP1 dimerization using the fluorescence assay developed by Fisher *et al.* (1999), which permits the study of dimeric interactions. In this assay, fluorescently labeled synthetic peptides interact in detergent solution and the interaction is measured by the level of FRET between a donor and an acceptor fluorophore. Homo- and heteromeric interactions were studied by diluting mixtures of these peptides in zwitterionic detergent micelles (LDAO). A reducing agent was added to avoid the formation of disulfide bridges during sample preparation. Figure 2B shows the results of the FRET studies for homo- and hetero-interactions between the different peptides in 5 mM LDAO. The only significant inter-

Figure 1. Predicted structural features of the NRP1 TM domain. (A) Helical-wheel representation of the dimer interface in the NRP1 TM domain sequence from Ile⁸⁵⁹ to Y⁸⁸⁰. The three glycine residues in the putative interface are indicated in bold characters and labeled with their residue number besides their one-letter amino acid codes. (B–D) A possible structure of the NRP1 TM domain homo-dimer. This TM domain dimer model was made by aligning the double GxxxG motif of the NRP1 TM sequence with the GxxxGxxxT dimerization motif of glycoprotein A (PDB code 1AFO; MacKenzie and Engelman, 1998), manually adjusting helices distance and angles, and by minimizing the energy of the dimeric structure to remove clashes using the Swiss-PDB viewer program (Guex and Peitsch, 1997). (B) A view down the dimer axis (C-terminus on top) shows the close packing of the glycine residues. (C) A view of the monomer shows the alignment of the three glycine residues (in red) on the same face of the helix. (D) A view along the dimer interface highlights the three glycines (in red) and the adjacent residues. These representations were made with the PyMol program (DeLano, 2002; <http://www.pymol.org>).

actions occurred between the NRP1 TM peptides. pTM-NRP^{mut} dimerized extremely weakly, if at all. Likewise, no significant FRET was observed for the interaction of pTM-NRP1 and pTM-NRP1^{mut}, and between the NRP peptides and the reference GPA TM peptide at concentrations up to 2 μ M. We also observed that pTM-NRP is unable to interact with peptides from the unrelated TM sequences of the epidermal growth factor and ErbB2 receptors (data not shown). These data allowed us to estimate the apparent K_d of ~ 120 nM, compared with ~ 60 nM for a GPA TM peptide. These data, together with the ToxLuc reporter assay, show that the NRP1 TM sequence is able to homodimerize. This capacity depends on the presence of a double GxxxG motif and the absence of nonspecific heterodimerization in the FRET assays supports a strong degree of specificity.

Figure 2. Transmembrane domain dimerization capacity. (A) Luciferase luminescence induced by several constructs. DH5 α , bacteria without construct; pcc, empty plasmid; GPA, glycoprotein A; NRP1, neuropilin 1. Student's *t* test analysis (* $p < 0.001$, ns, nonsignificant). (B) Homo- and heterodimer formation in detergent micelles. Initial samples were prepared with an equimolar mixture of donor (pyrene) and acceptor (coumarin)-labeled peptides at a total concentration of 2 μ M. Total peptide concentration was varied by a serial dilution in detergent buffer. Excitation spectra were collected, and pyrene (sensitized emission) and coumarin (direct emission) contribution to emission at 500 nm were measured and converted into the FRET ratio, which is proportional to the dimer fraction (see *Materials and Methods* for details). The dimer fraction was plotted as a function of total peptide concentration in semilogarithmic form. Homodimerization between pTM-NRP1 (●), pTM-NRP1^{mut} (○), pTM-GpA (Δ), and heterodimerization between pTM-NRP1 and pTM-NRP1^{mut} (peptides, ■), pTM-NRP1 and pTM-GpA (▲) as well as pTM-NRP1^{mut} and pTM-GpA (◇) are depicted. Results represent mean $\pm$ SEM of 2–4 independent experiments. The only significant dimerization occurred between pTM-NRP1 peptides and between the positive control pTM-GpA peptides.

Synthetic Peptides Derived from the NRP1 TM Antagonize Sema3A-induced Cortical Growth Cone Collapse

Sema3A acts as a strong axon growth inhibitory signal for many different neuronal types including cortical neurons (Fiore and Puschel, 2003). This inhibitory effect requires the binding of Sema3A to NRP1 (Castellani and Rougon, 2002). Previous work in our laboratory has demonstrated that synthetic peptides mimicking the TM domains are useful tools for investigating the biological function of such domains (Bennasroune *et al.*, 2004). We therefore performed a growth cone collapse assay to test the ability of pTM-NRP1 to block the activity of Sema3A. Primary cortical neurons were grown for 48 h in culture before Sema3A (100 ng/ml) was added for 2 h at a concentration inducing the collapse of more than 50% of the growth cones (Figure 3A). pTM-NRP1 suppressed the inhibitory effect of Sema3A in a dose-dependent manner (Figure 3A). In contrast, the addition of the

Figure 3. Functional study on embryonic cortical neurons. Explants of E15 neocortex were grown for 48 h to obtain a sufficient axonal growth to perform a collapse assay. Sema3A or combination of Sema3A and peptides were added to the culture medium for 2 h. For each explant, total number of growth cones and collapsed growth cones were counted. The percentage of growth cone collapse was calculated for each condition tested and statistical analyses were made by using χ^2 test analysis (* $p < 0.001$). (A) Effects of increasing concentrations of pTM-NRP1. (B) Effects of the mutated peptide at 10^{-8} M. (C) pTM-NRP1 blocking properties of Sema3A inhibitory effects were also tested at 10^{-8} M in DMSO as an alternative detergent vehicle. (D) Effects of pTM-ErbB2 (10^{-8} M), a NRP1-unrelated peptide containing a GxxxG motif. A minimum of 500 growth cones were analyzed in each conditions in at least three independent experiments.

mutated version of the peptide (pTM-NRP1^{mut}) failed to suppress Sema3A-dependent growth cone collapse (Figure 3B). Control experiments with a different vehicle for the pTM-NRP1 or with unrelated pTM confirmed the specificity of pTM-NRP1. Replacement of LDS by DMSO did not influence the ability of pTM-NRP1 to block the effects of Sema3A (Figure 3C). Despite the presence of a GxxxG motif, pTM-ErbB2 did not block Sema3A-induced growth cone collapse, therefore demonstrating a high specificity of pTM-NRP1 peptide (Figure 3D). Remarkably, the addition of pTM-NRP1 was also able to block Sema3A-induced repulsion of dorsal root ganglia axons (NGF-dependent) in coculture experiments (data not shown).

The Biological Function of NRP1 Requires the Integrity of the GxxxGxxxG Motif

COS cells do not express semaphorin receptors and are therefore not naturally sensitive to these guidance signals (Turner and Hall, 2006). However, the expression of NRP1 and PlexA1 in COS cells allows Sema3A to trigger a cellular collapse. This assay has been well documented and was used to evaluate the inhibitory property of Sema3A (Turner and Hall, 2006). Thus, we analyzed the percentage of cellular collapse of NRP1/PlexA1-expressing COS cells treated with Sema3A-conditioned medium in the presence or not of pTM-NRP1 (or pTM-NRP1^{mut}) to investigate if the peptides

Figure 4. Functional study on COS cells. COS cells coexpressing NRP1 or NRP1^{mut} and PlexA1 were incubated with control medium or Sema3A containing medium for 4 h. pTM-NRP1 and pTM-NRP1^{mut} were added at a concentration of 1 nM 1 h before treatments with Sema3A. In a collapse assay, percentage of cell collapse was determined for NRP1/PlexA1-expressing COS cells and for NRP1^{mut}/PlexA1-expressing cells. For each condition, 400 cells were counted and categorized as normal (spread cells) or collapsed cells (round cells). (χ^2 test analysis; * $p < 0.001$, ns, nonsignificant).

can block signaling by the Sema3A receptor. More than 40% of the cells treated with Sema3A collapsed, whereas only 10% of untreated cells were collapsed in control conditions (Figure 4). Strikingly, the addition of pTM-NRP1 completely abolished the effect of Sema3A when added at a concentration of 1 nM. The specificity of this blocking effect was confirmed by using the pTM-NRP1^{mut} peptide. Addition of pTM-NRP1^{mut} did not block the Sema3A-induced COS cell collapse. To confirm the pivotal role of the GxxxGxxxG motif, mutations were introduced into the TM domain of a full-length NRP1 to replace all three glycine residues by valines (NRP1^{mut}), as in the mutated peptide. When mutant NRP1 (TM-NRP1^{mut}) was expressed in COS cells together with PlexA1, Sema3A was no longer able to induce cell collapse (Figure 4). This further confirmed the importance of the GxxxGxxxG motif of NRP1 TM domain for the formation of a functional Sema3A receptor.

The GxxxGxxxG Motif Is Required But Not Sufficient to Ensure Ligand Binding to NRP1

To address the mechanism by which the TM NRP1 blocked Sema3A signaling, we performed binding assays. NRP1 expressing COS cells were incubated with AP-Sema3A, a fusion protein of Sema3A and the secreted AP (Bagnard *et al.*, 1998). After 2 h of incubation, binding was determined by the measurement of the luminescence of an AP substrate. The binding of AP-Sema3A was significantly reduced by addition of pTM-NRP1 (Figure 5). Strikingly, the addition of the mutated version of the pTM-NRP1, pTM-NRP1^{mut}, did not block AP-Sema3A binding to COS cells. To validate further the observed decrease of Sema3A binding when blocking TM NRP1, we decided to repeat binding experiments in COS cells expressing the mutated form of NRP1. We verified NRP1 and NRP1^{mut} expression rate by Western blots, using actin as a reporter protein for equal loading. Strikingly, this mutated form of the protein was still able to bind Sema3A, but quantitative analysis revealed a 44% reduction ($p < 0.001$) of binding, similar to the results obtained with synthetic peptides (Figure 5B). This result is consistent with other studies that have shown the role of the MAM domain of NRP1 for semaphorin binding and dem-

Figure 5. Binding experiments on COS cells. (A) NRP1 expressing COS cells (COS-NRP1) were incubated with pTM-NRP1 or pTM-NRP1^{mut} and then for 2 h at 4°C with conditioned medium containing AP-Sema3A. Bound AP-Sema3A was detected by addition of an AP luminescent substrate (Lumi-phos, Lumigen). (B) Binding experiments were performed on NRP1-expressing COS cells (COS-NRP1) and mutated NRP1-expressing COS cells (COS-NRP1^{mut}). (C) Similarly, the expression of the mutated NRP1 caused a significant reduction of VEGF binding. (Student's *t* test analysis; **p* < 0.05).

onstrates that the TM domain of NRP1 is not sufficient to mediate the binding of Sema3A. Thus, the specific blockade of TM NRP1 is able to inhibit totally the biological function of Sema3A without total inhibition of binding. Interestingly, the observed decreased of binding is ligand-independent because the mutated form of NRP1 exhibits reduced capacity of VEGF binding (Figure 5C).

Blockade of TM NRP1 Dimerization Prevents NRP1 Oligomerization

To further investigate the biochemical consequence of the TM NRP1 in terms of receptor complex formation, we analyzed the formation of complexes in COS cells expressing NRP1 and PlexA1. The COS cells were treated or not with Sema3A for 90 min before analysis of receptor complex formation in the presence of TM peptides. Collections of protein extracts were analyzed by centrifugation in sucrose gradient and Western blot analysis of NRP1 content in the different collected fractions. As seen in Figure 6, the addition of Sema3A triggered a shift of NRP1 to heavy fractions compare to control conditions, suggesting the formation of higher order oligomers. Strikingly, the Sema3A-induced NRP1 oligomerization was blocked by the addition of pTM-

Figure 6. Analysis of sedimentation constants by centrifugation in sucrose gradients. Confluent COS-1 cells expressing NRP1 or mutated NRP1 and PlexA1 were incubated with TM peptide for 1 h at 37°C. The culture medium was replaced by conditioned medium containing AP-coupled Sema3A. Cellular lysates were submitted to sucrose gradient centrifugation. Fractions were collected from the bottom and subjected to SDS-PAGE. For sedimentation constant(s) evaluation, the gradient was calibrated with thyroglobulin (19.3S), ferritin (17.7S), catalase (11S), lactate dehydrogenase (7S), and albumin (4.3S).

NRP1 but not by pTM-NRP1^{mut}. Control experiments showed that pTM-NRP1 or pTM-NRP1^{mut} alone had no effect on NRP1 distribution in the absence of Sema3A (data not shown). Thus, the inhibition of the TM domain of NRP1 is able to suppress NRP1 oligomerization and subsequent biological activity. Interestingly, we obtained similar results in a model of neuronal cells (PC12 cells) expressing multiple partners of Sema3A (data not shown).

DISCUSSION

Although the traditional view of the function of TM domains in bitopic membrane proteins has been mostly limited to membrane anchoring, recent studies have provided many examples for additional functions. For example, interactions between TM domains contribute to receptor dimerization and thus play a role in the activation of tyrosine kinase receptors of the ErbB family (Mendrola *et al.*, 2002; Benasroune *et al.*, 2004), the erythropoietin receptor (Constantinescu *et al.*, 2001), alphaIIb integrin (Li *et al.*, 2004), or the T-cell receptor (Teixeiro *et al.*, 2002). These interactions depend on the presence of sequence motifs that govern the packing of the TM helices into homo- or hetero-oligomers (Gordon-Weeks *et al.*, 1989; Arkin, 2002; Curran and Engelman, 2003; Senes *et al.*, 2004). Among these motifs, a short sequence composed of two glycines separated by any three amino acids is of particular interest, because it has been very extensively characterized in the case of GPA. It is the most frequent sequence motif in naturally occurring TM domains (Senes *et al.*, 2000) and is also the most frequent packing motif isolated in a randomized sequence library using the TOXCAT system (Russ and Engelman, 2000). Hence, this motif is encountered in helix interfaces in soluble proteins (Kleiger *et al.*, 2002). The observation that the TM domain of NRP1 contains a double GxxxG motif, and that this motif is conserved in all known NRP1 sequences (100% conservation in mammals, birds, fishes and amphibians) prompted us to hypothesize that this motif in the TM domain may be of functional importance in NRP1-related signaling. To test this hypothesis, we verified the propensity of this sequence to form dimers in a genetic assay and by FRET, we used

synthetic TM peptides in several different functional assays, and we demonstrated the role of the three glycine residues by a triple mutation both in synthetic peptides and in the full-length protein.

First, we could verify that the NRP1 TM sequence is indeed able to dimerize, both in reporter gene and FRET assays. The FRET assay allowed us to calculate an apparent K_d of 120 nM for pTM-NRP1 dimerization in our detergent conditions and clearly showed that the triple G→V mutant pTM-NRP1^{mut} had very little dimerization propensity. No K_d value could be determined for this mutant peptide because aggregates formed at concentrations higher than 2 μ M. Also, very importantly, the FRET system did not show any heteromeric interactions between the different peptides tested. Next, to address the function of the TM domain for signaling by the Sema3A receptor, we tested if the inhibition by TM peptides is able to block the effects of Sema3A in a collapse assay. This assay has been initially developed to assess the inhibitory properties of repellent factors on chick DRG axons (Raper and Kapfhammer, 1990) and has been widely used for many other types of neurons, including cortical neurons (Bagnard *et al.*, 1998). Here we used purified Sema3A (at a concentration of 100 ng/ml) in order to standardize the rate of observed growth cone collapse. Increasing concentration of pTM-NRP1 blocked the growth cone collapse induced by Sema3A in a dose-dependent manner. The reduction of the biological activity was not due to nonspecific effects on membrane composition or receptor interactions caused by the incorporation of the peptides because a mutated version of the peptide added at the same concentration was not able to reduce Sema3A collapsing activity. We chose to replace the three glycine residues in the GxxxG motifs by valines, as a compromise between the use of much larger amino acids such as phenylalanine, or the more conservative replacement by alanine, another small amino acid. Remarkably, the fact that pTM-ErbB2 was not able to block Sema3A collapsing activity despite the presence of a GxxxG motif strongly suggests that the presence of this motif is not sufficient to mediate dimerization. Rather, the entire sequence of the TM, the relative position of the double GxxxG motif and/or the nature of the other amino acids included in the motif may define the specificity of interactions. Further mutagenesis studies will be needed to clarify this issue. Nevertheless, the absence of signaling by the triply mutated full-length NRP1 proves the importance of this interaction motif.

The COS collapse assay is widely used to study the functional properties of semaphorin receptors (Turner and Hall, 2006). Wild-type COS cells are not sensitive to semaphorin because they do not express the appropriate receptors. After expression of both NRP1 and PlexA1, the COS cells collapse in response to Sema3A, a response that resembles axonal growth cone collapse. Thus, it is possible to perform biochemical analysis in a context of limited expression of semaphorin receptors. Both in COS cells and in cortical neurons, the addition of pTM-NRP1 but not pTM-NRP1^{mut} blocked the inhibitory effect of Sema3A. Moreover, the expression of a mutated full-length NRP1 confirmed that the alteration of the TM domain is sufficient to block Sema3A-dependent inhibitory effects. However, receptor affinity probe experiments revealed that Sema3A binding to NRP1 was reduced by the addition of pTM-NRP1 or when expressing the mutated form of NRP1 (NRP1^{mut}) but was not completely abolished. We hypothesized that the biological function of Sema3A was abrogated when interfering with the TM domain because of the destabilization of the receptor complex required for signal transduction. Indeed, it has been pro-

posed that Sema3A dimer (known to be important for binding and collapsing activity; Klostermann *et al.*, 1998; Koppel and Raper, 1998) may undergo a dimer-to-monomer transition upon binding to monomeric NRP1 (Antipenko *et al.*, 2003), a transition that would explain the persistence of binding after destabilization of NRP1 homodimerization. In the other hand, the interference of the TM domain dimerization may be sufficient to generate conformational changes altering optimal binding at the extracellular level. Hence, there has been much discussion about the dimerization capacity of NRP1. Initial studies performed in 1998 (Giger *et al.*, 1998; Nakamura *et al.*, 1998) proposed that the MAM domain of NRP1 is important for dimerization and oligomerization. The use of chimeric NRP1 in which the TM and cytoplasmic tail were replaced by the corresponding segments of NRP2 or L1 and GPI-anchored recombinant NRP1 (lacking TM and cytoplasmic domains) suggested that the TM domain and intracellular domain were not required for multimerization, although oligomerization still occurred in mutants presenting deletion of the MAM domain (Nakamura *et al.*, 1998). Moreover, it appeared that the MAM domain in conjunction with the TM domain of NRP1 was sufficient to mediate multimerization, whereas the MAM domain alone or the TM/cytoplasmic domain alone appeared incapable of multimerization (Giger *et al.*, 1998). These studies suggested that multiple domains are required for oligomerization of neuropilins. Our results provide evidence for a critical role of the TM domain at least during homodimerization of NRP1 or oligomerization. The monitoring of NRP1 distribution in the different fractions obtained in sucrose gradients experiments allowed us to attribute the observed functional blockade to a destabilization of NRP1 oligomerization. First, our data confirmed that NRP1 dimers preexist at the cell surface in the absence of ligand as previously suggested (Takahashi *et al.*, 1998). Second, we found that Sema3A triggered NRP1 oligomerization because NRP1 was redistributed in heavy fractions of the gradients upon ligand binding. Although calculated sedimentation constants are approximate because only partial crystal structure of the protein has been resolved (Antipenko *et al.*, 2003), oligomerization is evident from our results and may possibly be underestimated. This further supports the conclusion that both MAM and TM domain interactions are necessary for the induction of neuropilin signaling, possibly in a concerted manner, because both types of interactions can be effectively antagonized. The exact structural mechanism requires further exploration, especially to establish the composition and organization of higher order membrane complexes necessary for neuropilin signaling. Future studies will investigate how heterodimerization and/or oligomerization may be dependent on the GxxxG motifs encountered in the TM domains of multiple receptors of semaphorins including NRP2, PlexA1, L1, NrCAM, or integrins.

Currently, NRP1 has been implicated in a variety of physiological and pathological processes ranging from nervous system development, angiogenesis, to cancer biology. Hence, our results not only shed light on the primary molecular events participating in receptor dimerization and oligomerization but also offer a novel strategy to interfere with NRP1 signaling. Recently, peptidic antagonists of NRP1 have been successfully used to block Sema3A signaling (Williams *et al.*, 2005). Moreover, function blocking antibodies to NRP1 reduce tumor growth (Pan *et al.*, 2007), and selective inhibitors of Sema3A improve nerve regeneration (Kaneko *et al.*, 2006). The hydrophobic character of the pTM-NRP1 offers a unique chance to target naturally the heart of the cell membrane and thereby to increase the probability of inter-

action with the protein domains to modulate. The use of hydrophobic peptides directed against the GxxxG motifs of TM helices may allow the design of new reagents to probe the role of such hydrophobic domains in membrane protein assembly and signal transduction. These may also lead to the development of new therapeutic approaches. A very elegant work has recently shown that it is possible, through computational methods, to design peptides that target TM helices in a very specific manner (Yin *et al.*, 2007).

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