Semaphorin4F interacts with the synapse-associated protein SAP90/PSD-95

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Abstract

Semaphorins are a family of secreted and membrane-associated proteins involved in growth cone guidance during development. Here, we describe the interaction of Semaphorin4F (Sema4F) with the post-synaptic density protein SAP90/PSD-95. Using the yeast two-hybrid system and coprecipitation assays we were able to show an interaction between the extreme C-terminus of Sema4F and the PDZ domains of SAP90/PSD-95. Heterologous coexpression of a chimeric EphrinB1/Semaphorin4F protein with SAP90/PSD-95 in COS cells leads to translocation of SAP90/PSD-95 from the cytosol to the membrane. Deletion analysis shows that this translocation activity of Sema4F is completely dependent on the presence of the last three C-terminal amino acids. In addition, Sema4F immunoreactivity is present in synaptosome fractions and enriched in post-synaptic density fractions. Consistently, in cultured hippocampal neurons, we demonstrate punctate colocalization of Sema4F and SAP90/PSD-95 in dendrites, furthermore we found colocalization of Sema4F with synapsin1 suggesting a synaptic localization. Our data implicate a new functional context for semaphorins at glutamatergic synapses.

Keywords: path finding, post-synaptic density, PSD-95, SAP90, semaphorin, synapse, synaptic plasticity.

The semaphorins are a large family of secreted and membrane-bound proteins that are characterized by a conserved *N*-terminal located domain of ≈ 500 amino acids, the sema domain (Kolodkin *et al*. 1993; Yu and Kolodkin 1999; Nakamura *et al*. 2000; Tamagnone and Comoglio 2000). According to their modular structure and membrane-binding capacity, semaphorins are divided into eight different classes (Semaphorin Nomenclature Committee 1999). Semaphorins play important roles in axon guidance, in the regulation of cell migration and angiogenesis (Behar *et al*. 1996; Kitsukawa *et al*. 1997), and in the modulation of the immune system (Hall *et al*. 1996; Delaire *et al*. 1998). Recent evidence shows additional functions of semaphorins in regulating neuronal apoptosis (Gagliardini and Fankhaus 1999; Shirvan *et al*. 1999) and metastatic growth of tumours (Yamada *et al*. 1997; Christensen *et al*. 1998). However, the function of semaphorins is best characterized as ensuring the correct connectivity of the nervous system during development (Mark *et al*. 1997). Only recently has there been major progress in identifiying the signal transduction mechanisms of semaphorins. First, the neuropilins were identified as transmembrane molecules binding class 3 semaphorins (Chen *et al*. 1997; He and Tessier-Lavigne 1997; Kolodkin *et al*. 1997). However, the cytoplasmic domain of neuropilin-1 is not necessary for Sema3A activity and consequently a second class of transmembrane semaphorin receptors was identified, the plexins (Ohta *et al*. 1995; Comeau *et al*. 1998). Molecular and functional interactions of neuropilin and plexins could be demonstrated suggesting a heteromeric receptor for semaphorins composed of

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Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; PDZ, post-synaptic density protein-95, discs large protein, zonula occludens; PSD-95, post-synaptic density protein-95; SAP90, synapse associated protein 90; SDS−PAGE, sodium dodecyl sulphate−polyacrylamide gel electrophoresis; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

neuropilins and plexins (Takahashi *et al*. 1999; Tamagnone *et al.* 1999; Rohm *et al.* 2000).

Among the eight classes of semaphorins, there are four (classes 1, 4, 5 and 6) that contain a transmembrane domain and a short cytoplasmic tail. Little is known about the physiological role of these transmembrane semaphorins, however, in support of a bidirectional signalling system, mouse Sema6B associates with the tyrosine kinase c-*src* (Eckhardt *et al*. 1997), and mouse Sema4D forms a complex with a serine kinase (Elhabazi *et al*. 1997). Moreover, Sema4C is able to interact with SEMCAP1/GIPC, a PDZ domain-containing protein (Wang *et al*. 1999). These interactions may lead to a different signal transduction pathway, as proposed for the ephrins, and/or may regulate the subcellular distribution of semaphorins. The functions of semaphorins during development are well established, but little is known about their function postnatally, although expression of Sema4F for example increases significantly, even after birth (Encinas *et al*. 1999). Here, we describe the interaction of the transmembrane semaphorin Sema4F with the post-synaptic density protein SAP90/PSD-95 (Cho *et al*. 1992; Kistner *et al*. 1993). We demonstrate the capacity of the cytoplasmic domain of Sema4F to interact with the PDZ domains of SAP90/PSD-95 and characterize this interaction biochemically. In addition, we show colocalization of both proteins in hippocampal neurons, suggesting a new role for semaphorins in the regulation of glutamatergic synapses.

Material and methods

Expression vectors

The C-terminal part of Sema4F (amino acids 698–777) was cloned from mouse brain using reversed PCR with the primer (up) GATGAATTCGAGCTTCTAGCTAGAGACAAG and primer (down) TAGGGATCCTTAGATAGACTGCTCATCGCA (amplifying nucleotides 2170–2412; Encinas *et al*., 1999) and subcloned into yeast expression vector pGBT9 vector (Clontech, Heidelberg, Germany). To construct EphrinB1/Sema4F the cytoplasmic and transmembrane domain of Sema4F (amino acids 670–777, nucleotides 2088–2412) was amplified using the primer TTCTTC AACTCCAAGACCGTTGTGGGGGCTGGGCTG and the primer up from a cDNA clone representing almost the complete coding sequence of mouse Sema4F isolated from a screen of a mouse brain cDNA library (Clontech). The extracellular domain of mouse EphrinB1 (amino acids 1–236, nucleotides 689–1426; Bouillet *et al*. 1995) was amplified by PCR using the primer ACGGGT ACCGATCCTGAAGTGCATTCTGCC and AGCCCCCAC AACGGTCTTGGAGTTGAAGAAGCT. The two overlapping fragments were combined by splicing by overlapping ends (SOE)−PCR (Horton *et al*. 1989). The resulting cDNA construct was cloned into the pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). For the SAP90/PSD-95 expression construct the entire coding sequence (amino acids 1–725, nucleotides 203–2380) was amplified from a plasmid containing full-length SAP90 (Kistner *et al*. 1993) using primer 1 CATG AATTCGTCGCCACCATGGACTGTCTCTGTATAGTGAC and primer 2 GATCTCGAGATCAGAGTCTCTCTCGGGCTG and subcloned into pcDNA3.

Yeast two-hybrid system

The C-terminal last 240 nucleotides corresponding to the entire cytoplasmic domain of mouse Sema4F cDNA sequence were subcloned into the pGBT9 vector (Clontech). Yeast strain Hf7c (Clontech) was transformed sequentially with pGBT9Sema4F and a mouse brain cDNA library (Clontech). To identify the interacting PDZ domains and the specificity of this interaction, the cDNA regions corresponding to each of the three PDZ domains of rat SAP90/PSD-95 were amplified by PCR and subcloned into the pGADGH vector (Clontech). Cotransformants were selected on minimal agar plates lacking leucine, tryptophan and histidine. For the β-galactosidase assay cotransformants were plated on plates lacking leucine and tryptophan, filter-lifts were performed and incubated with 40 μg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) in buffer Z.

Antibodies

AntiSema4F antibodies were raised in rabbits against the peptide SGTTSYSQDPPSPSPEDER corresponding to amino acids 708–732 of mouse amino acid sequence of Sema4F. The antibody was affinity purified using an antigen-coupled affinity column (Affigel-10, Bio-Rad, Germany). A monoclonal antiSAP90/PSD-95 antibody (7E3-1B8) was purchased from Dianova, Germany. An Alexa488-conjugated anti-rabbit antibody was purchased from Molecular Probes (Eugene, OR, USA), anti-mouse Cy3 and anti-human fluorescein isothiocyanate (FITC)-labelled antibodies were from Sigma-Aldrich (Taufkirchen, Germany). EphB1−Fc containing the extracellular domain of the EphB1 receptor fused to the Fc-part of a human IgG was purchased from R & D Systems (Minneapolis, MN, USA).

Immunocytochemistry

Hippocampal cells

Hippocampal neurons from new-born (P0-P4) Wistar rats were prepared as described previously (Haubensak *et al*. 1998). Neurons were plated on poly(D-ornithine)-coated (0.5 mg/mL overnight) glass coverslips at a density of 300 000–400 000 cells/3.5 cm culture dish in Neurobasal medium/B27 supplement without serum, 5% CO_2 atmosphere. Cultures were supplemented with 4 μM arabinofuranoside (ARAC) after 4 days *in vitro*. Neurons were fixed with 4% paraformaldehyde containing 4% sucrose for 15 min on ice. Extensive paraformaldehyde was quenched by incubation with 0.1 M glycine in phosphate-buffered saline (PBS; 65 mM NaCl, 2.5 mM KCl, 1.2 mM $NaHCO_3$, 2 mM $KHCO_3$, pH 7.4). After incubation with 0.5% NP-40 in PBS for 15 min, cells were washed four times for 8 min with PBS. Unspecific binding was blocked by 1% bovine serum albumin in PBS for 15 min at room temperature (22°C). Cells were incubated for 1 h with affinity-purified anti-Sema4F (1:30) and anti-PSD-95 (1:200) or anti-Synapsin1 (1:200) antibody. First, antibodies were detected using an ALEXA488-conjugated anti-rabbit or a Cy3-conjugated anti-mouse antibody. After each incubation the coverslips were washed four times for 8 min with PBS. The coverslips were mounted using Immunoflor (ICN Biomedicals, Aurora, OH, USA) to prevent bleaching.

COS cells

COS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum at 37°C in a 10% CO_2 atmosphere. Cells (20 000–50 000) were plated on poly(D-ornithine)-coated coverslips and transfected with a plasmid coding for SAP90/PSD-95 or the chimeric protein EphrinB1/Sema4F, as indicated, using the calcium phosphate method. Between 16 and 20 h after transfection coverslips were washed twice with medium and incubated for an additional 48 h. Transfected cells were fixed with 4% paraformaldehyde. Extensive paraformaldehyde was quenched by incubation with 0.1 M glycine. After blocking of unspecific binding by incubation with 1% bovine serum albumin (BSA) cells were incubated for 1 h with EphB1–Fc (1:35) for extracellular labelling of the chimeric protein. Subsequently, cells were permeabilized with 0.5% NP-40, 1% BSA and incubated with anti-PSD-95 antibody (1 : 200) for 1 h. Pictures were taken with a CCD camera (Sensys 1401E) mounted to an Olympus IX70 inverted microscope or with a Leica TCS 4D confocal microscope.

Coprecipitation and isolation of synaptosomes and PSD fraction

Synaptosomes and PSD fractions were isolated from total juvenile rat brain as described by Carlin *et al*. (1980). For coprecipitation experiments 6×10^5 COS cells were cultured on a 10-cm culture dish. After 10 h, the cells were transfected with plasmids encoding for SAP90/PSD-95 and EphrinB1/Sema4F or EphrinB1/Sema4FΔ using the calcium phosphate method (Chen and Okayama 1987). After incubation overnight, medium was removed, the cells were washed once with PBS and new medium was added. After an additional 48 h incubation, the cells were washed twice with 10 mL cold PBS and lysed in 600 μL lysis buffer (0.2% NP-40, 100 mM NaCl, 1 mM $MgCl_2$, 20 mM Tris/HCl pH 7.4, 1 mM phenylmethylsulfonylfluoride, 10 μg/mL aprotinin). Lysates were cleared by centrifuging twice for 10 min (14 000 *g*, 4°C); 10 μg of EphB1–Fc was added. After incubation with rotation at 4°C for 1 h, 30 μL protein A–Sepharose was added and incubation was continued for an additional 4 h. Beads were pelleted and washed four times with lysis buffer. Finally, proteins were eluted with 30 μL of Laemmli sample buffer and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a nitrocellulose membrane by electroblotting. After incubation with the respective antibody, blots were developed using the ECL system (Amersham Pharmacia Biotech, Germany). Anti-SAP90/PSD-95 was diluted 1 : 200 and anti-Sema4F was diluted 1 : 200 for immunoblotting. For immunoprecipitation from the PSD fraction, 30 μg of PSD fraction was solubilized at room temperature in 2% SDS in IP-buffer (Lau *et al*. 1996; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 5 mM EDTA, 5 mM EGTA, 1 mM $NaVO_4$, 50 mM NaF and 1× inhibitor cocktail; Roche Diagnostics, Germany) for 20 min, the mixture was diluted with 5 vol. of ice-cold 2% Triton in IP-buffer. After addition of 15 μL anti-sema4F serum or 15 μL pre-immune serum the mixture was incubated for 1 h with rotation at 4°C. Fifty microlitres of protein A–Sepharose (slurry 1 : 1) was added and the incubation was prolonged for an additional 4 h. Precipitates were washed four times with IP-buffer and proteins were eluted in 1× Laemmli sample buffer. Proteins were fractionated on 8% SDS–PAGE and transferred to nitrocellulose. PSD-95 was detected as described above.

Results

Sema4F interacts via its extreme C-terminus with the PDZ domains of SAP90/PSD-95

The cytosolic domain of Sema4F is highly conserved between mouse, rat and human suggesting an important role for this domain in Sema4F function (Fig. 1a). To identify proteins that interact with Sema4F, we applied the yeast two-hybrid system using the entire cytoplasmic domain as a bait to screen a mouse brain cDNA library. Among 10^6 screened primary transformants, we found five specifically interacting clones. One clone contained almost the complete coding sequence of the post-synaptic density protein SAP90/PSD-95. We analysed the interaction of Sema4F with each of the three PDZ domains of SAP90/PSD-95 using the yeast two-hybrid system and found specific interaction with all three PDZ domains, although

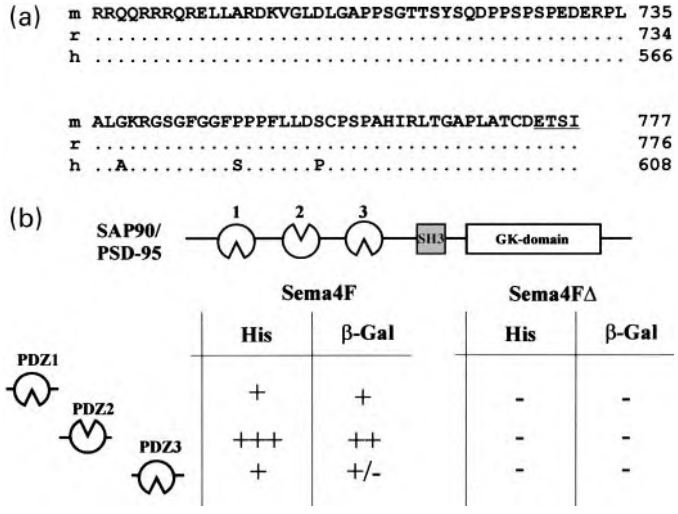

Fig. 1 (a) Alignment of the cytoplasmic domain of mouse (m), rat (r) and human (h) Sema4F amino acid sequences, the PDZ-binding motif is underlined. (b) Yeast two-hybrid analysis of the interaction between Sema4F and SAP90/PSD–95. Interaction of the cytoplasmic domain of wild-type Sema4F or Sema4FΔ (a C-terminal deletion construct lacking the last three amino acids) with PDZ1, -2 and -3 of SAP90/PSD-95, as determined by two-hybrid assays. Interaction is indicated by histidine prototrophy (+) and β-galactosidase-activity (+); GK, guanylate kinase homology domain.

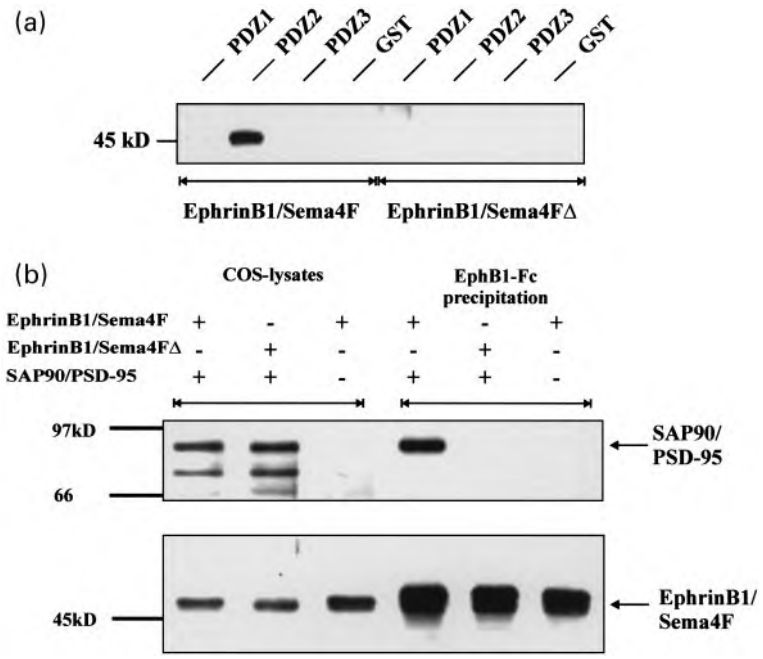

Fig. 2 (a) PDZ2 of SAP90/PSD-95 is able to precipitate a chimeric EphrinB1/Sema4F protein. COS cells were transfected with an expression vector for EphrinB1/Sema4F. Forty-eight hours after transfection lysates were incubated with the indicated glutathione-*S*-transferase fusion proteins. Fusion proteins were collected using glutathione−Sepharose beads and the precipitates were separated by 10% SDS−PAGE and transferred to nitrocellulose. The blot was developed using an antiSema4F antibody. (b) EphrinB1/Sema4F binds full-length SAP90/PSD-95. COS cells were cotransfected with an expression vector for SAP90/PSD-95, EphrinB1/Sema4F or EphrinB1/Sema4FΔ as indicated. Lysates were incubated with ephB1−Fc. Complexes were collected using protein A−Sepharose beads. Proteins were seperated by 10% SDS−PAGE and transferred to nitrocellulose. The blot was developed using an anti-SAP90/PSD-95 antibody (upper). The blot was stripped and redetected using an anti-Sema4F antibody (lower).

the strongest interaction seems to be with the PDZ2 domain as judged by histidine prototrophy and X-Gal staining (Fig. 1b). Because the C-terminus of Sema4F contains a known PDZ domain-binding motif, ETXI, we sought to determine the specificity of the interaction by deleting the last three amino acids of the cytosolic domain of Sema4F (Songyang *et al*. 1997). The deletion led to complete abolishment of the interaction with each of the PDZ domains. For further biochemical characterization we generated glutathione-*S*-transferase fusion proteins with the PDZ domains of SAP90/PSD-95 and performed pull-down assays using COS cell lysates transfected with a chimeric protein consisting of the extracellular domain of EphrinB1 and the transmembrane and cytoplasmic domain of Sema4F (EphrinB1/Sema4F). In line with our two-hybrid analysis, PDZ2 was able to pull down the chimeric receptor from the lysates. No precipitation was observed with PDZ1 and PDZ3 or GST alone. In addition, a deletion construct of EphrinB1/Sema4F (EphrinB1/Sema4FΔ) devoid of the last three C-terminal amino acids of Sema4F could not be coprecipitated with the PDZ domains (Fig. 2a).

Sema4F interacts with full-length SAP90/PSD-95

To establish additional evidence for a biochemical interaction between Sema4F and full-length SAP90/PSD-95, we analysed lysates of transfected COS cells for co-immunoprecipitation of both proteins. We transfected COS cells transiently with expression constructs for SAP90/PSD-95 and EphrinB1/Sema4F protein or EphrinB1/Sema4FΔ. Lysates of transfected cells were subjected to immunoprecipitation using the extracellular domain of ephB1 fused to the Fc part of a human immunglobulin (ephrinB1−Fc), which is able to bind the ephrinB1 extracellular domain of the EphrinB1/Sema4F chimeric protein. Only the chimeric protein containing the wild-type Sema4F C-terminus was able to coprecipitate SAP90/PSD-95, whereas deletion of the last three amino acids led to complete abolishment of coprecipitation (Fig. 2b). These results are in line with our two-hybrid and pull-down assays.

Sema4F translocates SAP90/PSD-95 from the cytosol to the membrane in heterologous transfected COS cells

To analyse whether an interaction of Sema4F and SAP90/PSD-95 can be observed in intact cells, we cotransfected COS cells with expression contructs for SAP90/PSD-95 and EphrinB1/Sema4F or EphrinB1/Sema4FΔ. Cells were stained for SAP90/PSD-95 and the chimeric Sema4F with anti-PSD-95 and ephB1−Fc, respectively. Using confocal imaging, we observed a plasma membrane-associated staining for the chimeric EphrinB1/Sema4F protein and EphrinB1/Sema4FΔ protein. However, efficient translocation of SAP90/PSD-95 from the cytoplasm to the membrane was detected only after cotransfection with EphrinB1/Sema4F. These results show that the interaction between Sema4F and SAP90/PSD-95 can be observed within cells and depends on the last three amino acids of Sema 4F (Fig. 3).

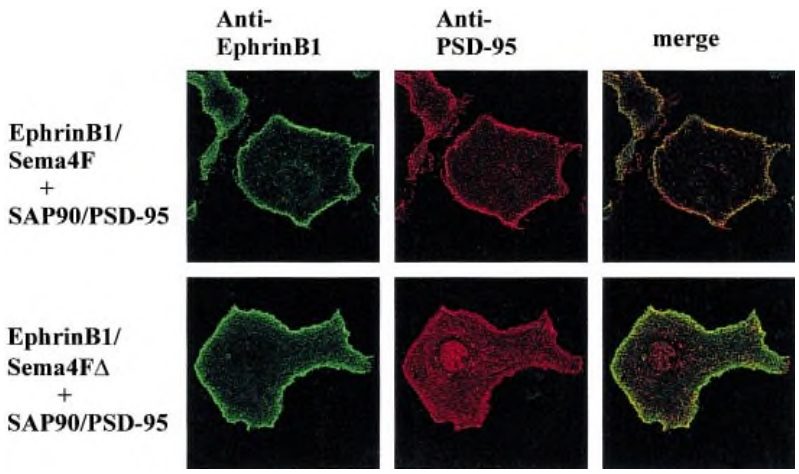

Fig. 3 EphrinB1/Sema4F, but not EphrinB1/Sema4FΔ, is able to translocate SAP90/PSD-95 from the cytosol to the membrane. COS cells were transfected with expression vectors for SAP90/PSD-95 and EphrinB1/Sema4F or EphrinB1/Sema4FΔ. Forty-eight hours after transfection cells were fixed and stained for the indicated proteins, bar = 10 μm.

Sema4F is present in synaptosome and post-synaptic densitiy fractions from rat brain

SAP90/PSD-95 is a major component of the post-synaptic density (PSD) fraction. We thus isolated synaptosomes and post-synaptic densities from rat brain to look for the presence of Sema4F in these fractions. Using an affinity-purified antibody raised against Sema4F we detected a doublet band in the 90-kDa range in the synaptosome fraction, which could be competeted with the peptide used for immunization (Fig. 4a). These bands were clearly increased in the corresponding post-synaptic density fraction, in addition a third band, which could be competeted became detectable in the PSD fraction at 97 kDa. These three bands probably represent different splicing variants of Sema4F (Encinas *et al*. 1999). For comparison, we analysed our fractions for the presence of SAP90/PSD-95. As expected, SAP90/PSD-95 was easily detected in the synaptosome fraction and strongly enriched in the post-synaptic density fraction.

To test whether SAP90/PSD-95 associates with endogenous Sema4F in rat brain, SAP90/PSD-95 was immunoprecipitated from solubilized rat brain PSD fractions using an antibody against Sema4F, no precipitation of SAP90/PSD-95 was detectable using the corresponding pre-immune serum (Fig. 4b).

Colocalization of Sema4F with SAP90/PSD-95 and synapsin1 in cultured hippocampal neurons

SAP90/PSD-95 is known to be enriched at glutamatergic synapses. Therefore, to analyse the subcellular localization

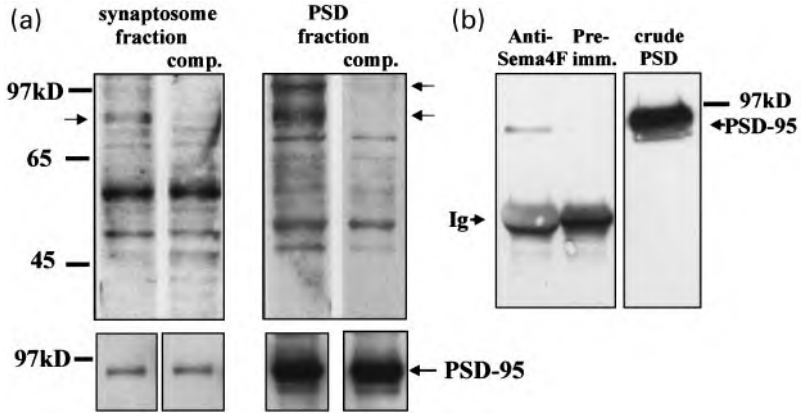

Fig. 4 (a) Sema4F immunoreactivity is present in synaptosome fractions and enriched in post-synaptic density fractions from rat brain. A 20-μg synaptosome fraction or 5-μg post-synaptic density fraction was separated on a 8% SDS polyacrylamide gel and transferred to nitrocellulose. The blot was developed using anti-Sema4F antibody. The blot was stripped and redetected with anti-SAP90/PSD-95 antibody. For competition a duplicate blot was detected with an anti-Sema4F or anti-SAP90/PSD-95 antibody pre-adsorbed with the peptide used for immunization. (b) Co-immunoprecipitation of SAP90/PSD-95 from rat brain. Solubilized rat brain PSD fractions were incubated with anti-Sema4F serum or the corresponding pre-immune serum. Immunoprecipitates were fractionated on SDS−PAGE and transferred to nitrocellulose. The blot was developed using an anti-PSD-95 antibody, the band labelled with Ig represents a cross-reactivity of the immunoglobulins used for immunoprecipitation, for comparison 10 μg solubilized PSD fraction was loaded (crude PSD).

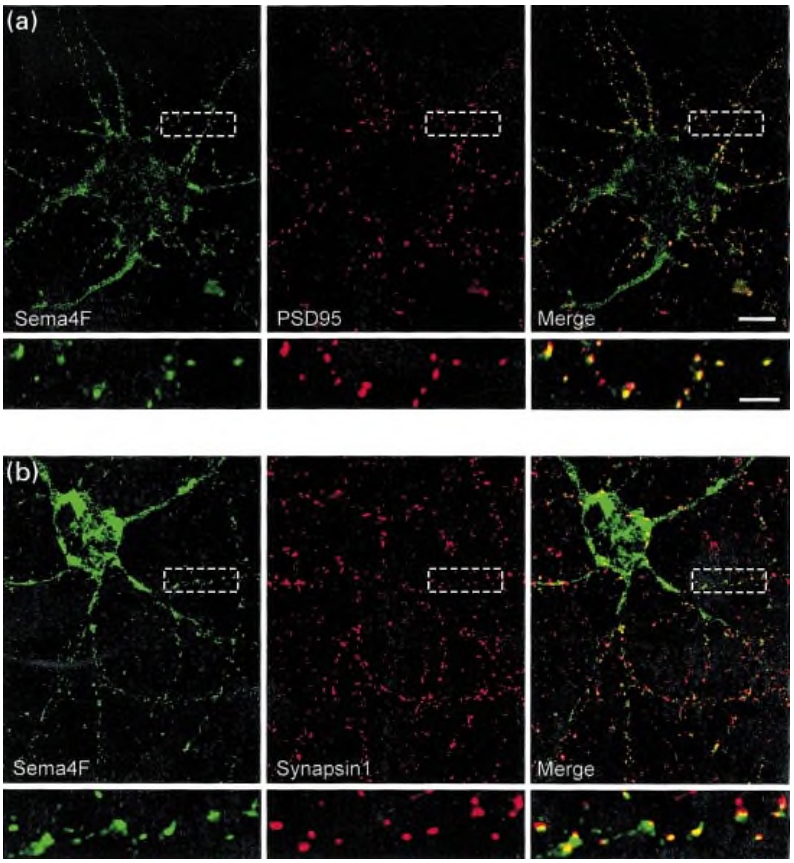

Fig. 5 Colocalization of Sema4F with SAP90/PSD-95 and synapsin1 in cultured hippocampal neurons. (Upper) Cultured hippocampal neurons were stained for endogenous Sema4F (green) and SAP90/PSD-95 (red) (a) or for endogenous Sema4F (green) and Synapsin1 (red) (b), bar = 10 μm. (Lower) Magnification of the marked area in the upper panels, bar = 3 μm.

of Sema4F in neurons we performed immunocytochemistry in cultured hippocampal neurons. We observed a high degree of colocalization of Sema4F and SAP90/PSD-95 in punctate structures, which outlined the processes (Fig. 5a). Similar colocalization was observed for Sema4F and a second synaptic marker protein (synapsin1, Fig. 5B), suggesting synaptic colocalization of SAP90/PSD-95 and Sema4F.

Discussion

The results presented here describe a novel interaction between the semaphorin Sema4F and the post-synaptic density protein SAP90/PSD-95. Yeast two-hybrid screening of a mouse cDNA library with the cytosolic domain of Sema4F identified SAP90/PSD-95 as a potential interaction partner for Sema4F. SAP90/PSD-95 is known to bind a number of proteins via its PDZ domains. We noticed that Sema4F contains a known C-terminal PDZ domain-binding consensus sequence (Songyang *et al*. 1997). Consistent with this observation, we demonstrated that deletion of the last three amino acids of Sema4F leads to a complete abolishment of the interaction with SAP90/PSD-95, as measured by yeast two-hybrid analysis and coprecipitation assays (Figs 1, 2). However, pull-down assays using glutathione-*S*-transferase fusion proteins of each PDZ domain of SAP90/PSD-95 revealed a specific interaction of Sema4F with PDZ2, but not with PDZ1 or PDZ3. This partially contrasts with our data obtained using the two-hybrid system, in which all three PDZ domains of SAP90/PSD-95 showed a specific interaction with the cytosolic domain of Sema4F and might be because of the different sensitivities of the assay systems.

In order to analyse whether the interaction between Sema4F and SAP90/PSD-95 also occurs in cells, we performed double immunocytochemistry on heterologous transfected COS cells expressing SAP90/PSD-95 and a chimeric protein consisting of the extracellular domain of EphrinB1 and the transmembrane and cytosolic domain of Sema4F (EphrinB1/Sema4F). We observed a translocation of the SAP90/PSD-95 protein from the cytosol to the membrane exclusively after cotransfection with EphrinB1/Sema4F but not with EphrinB1/Sema4FΔ, a deletion construct lacking the last three C-terminal amino acids. This indicates that the interaction between Sema4F and SAP90/PSD-95 is stable within cells and that the last three amino acids of Sema4F are essential for the interaction. Moreover, we were able to identify Sema4F immunoreactivity in isolated synaptosome and post-synaptic density fractions. Sema4F immunoreactivity was clearly enriched in the PSD fraction compared with the synaptosome fraction. In addition, we were able to immunoprecipitate SAP90/PSD-95 from solubilized

rat brain PSD fractions using an anti-body against Sema4F but not with the corresponding pre-immune serum. The presence of Sema4F in the synaptosome as well as in the post-synaptic density fraction suggests a synaptic localization for Sema4F. However, an additional extrasynaptic localization is not excluded.

We then analysed the subcellular localization of both proteins in cultured hippocampal neurons. We observed colocalization of Sema4F with SAP90/PSD-95 in dot-like structures, suggesting synaptic colocalization of both proteins. In addition, we observed colocalization of Sema4F with the pre-synaptic marker protein synapsin1, providing additional evidence of a synaptic localization for Sema4F. Pre-adsorption of the anti-Sema4F antibody with the peptide used for immunization greatly reduced the staining for Sema4F (data not shown).

The interaction between Sema4F and SAP90/PSD-95 may have several functional consequences for Sema4F. First, the interaction with SAP90/PSD-95 may lead to synaptic localization of Sema4F, as suggested by our double-immunofluorescence analysis of cultured hippocampal neurons. Second, similar to the ephrinB/eph-receptor system, PDZ domain interactions may lead to a clustering of Sema4F, which could influence its signal transduction (Wong *et al*. 1999). Although we did not observe clustering of our EphrinB1/Sema4F construct after coexpression with SAP90/PSD-95, Sema4F could associate with pre-clustered SAP90/PSD-95, as shown previously for fascilin II, which is recruited into the synapse by the *Drosophila* SAP90/PSD-95 homologue Discs-Large (Thomas *et al*. 1997). Sema4F may modulate synaptic function by exerting destabilizing effects as described previously for Sema-2a (Matthes *et al*. 1995) as well as stabilizing effects on synapses. However, in addition to a possible modulation of exisiting synapses, Sema4F could also be important for the development of synapses as proposed recently for the neuroligins (Scheiffele *et al*. 2000). Interestingly, neuroligins are also able to bind to SAP90/PSD-95 (Irie *et al*. 1997). In conclusion, the novel interaction described here between Sema4F and SAP90/PSD-95 suggests a possible role for semaphorins in the regulation of glutamatergic synapses.

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