The Neural Cell Recognition Molecule Neurofascin Interacts with Syntenin-1 but Not with Syntenin-2, Both of Which Reveal Self-associating Activity* 

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Neurofascin belongs to the L1 subgroup of the immunoglobulin superfamily of cell adhesion molecules and is implicated in axonal growth and fasciculation. We used yeast two-hybrid screening to identify proteins that interact with neurofascin intracellularly and therefore might link it to trafficking, spatial targeting, or signaling pathways. Here, we demonstrate that rat syntenin-1, previously published as syntenin, mda-9, or TACIP18 in human, is a neurofascin-binding protein that exhibits a widespread tissue expression pattern with a relative maximum in brain. Syntenin-1 was found not to interact with other vertebrate members of the L1 subgroup such as L1 itself or NrCAM. We confirmed the specificity of the neurofascin-syntenin-1 interaction by ligand-overlay assay, surface plasmon resonance analysis, and co-localization of both proteins in heterologous cells. The COOH terminus of neurofascin was mapped to interact with the second PDZ domain of syntenin-1. Furthermore, we isolated syntenin-2 that may be expressed in two isoforms. Despite their high sequence similarity to syntenin-1, syntenin-2α, which interacts with neurexin I, and syntenin-2β do not bind to neurofascin or several other transmembrane proteins that are binding partners of syntenin-1. Finally, we report that syntenin-1 and -2 both form homodimers and can interact with each other.

Neurofascin is a member of the L1 subgroup of the immunoglobulin superfamily of cell adhesion molecules, which also includes L1 (NgCAM) itself, CHL1, NrCAM in vertebrates, neuroglian in insects, and tracitin in leeches. These transmembrane glycoproteins share a well conserved overall domain organization with six immunoglobulin-like and four to five fibronectin type III (FNIII)-like domains. Their diverse homogeneous interactions mediate cell-cell contacts and can promote neuronal migration, axonal growth, and fasciculation in the developing nervous system (1–3). A crucial role of the L1 subgroup in neural development is exemplified by a range of neuroanatomical and neurological disorders caused by knock-out of the murine L1 gene (4–6) and by mutations in the human L1 gene, which affect L1 binding activity and trafficking (7, 8).

Unlike L1 and other subgroup members, neurofascin is subjected to extensive alternative splicing that is regulated during embryonic development of the chicken brain (9). This differential splicing has been shown to modulate interactions of neurofascin with axonal NrCAM, F11, axonin-1, and the extracellular matrix protein tenasin-R, and to influence neurite extension in vitro (10, 11). Specific isoforms of neurofascin are localized to initial axon segments of Purkinje cells and to the nodes of Ranvier of myelinated nerves, where they interact with the cytoskeleton adapter-protein ankyrin-G (12). In particular, an oligodendrocyte-specific form of neurofascin (termed NF155) was found to localize to the paranodal region, whereas a neuron-specific form (NF186) was confined to the nodal region (13, 14). Ankyrin binding appears to be a common feature of all L1-type molecules and is thought to stabilize cell adhesion (15–18). Interaction with ankyrin requires a highly conserved sequence within the cytoplasmic tails of L1 subgroup members and is inhibited by its tyrosine phosphorylation as demonstrated for neurofascin (19–21). Furthermore, palmitoylation of neurofascin at a highly conserved cysteine residue in its membrane-spanning segment might affect the targeting of neurofascin to specialized plasma membrane microdomains (22). L1CAM-mediated cellular processes may also be regulated by changes in the expression levels of CAMs on the cell surface. Tyrosine phosphorylation of the endocytic motif YRSL, which represents a binding site of the AP-2 clathrin adaptor complex, regulates not only the internalization of the neuronal L1 form but probably also of NrCAM and neurofascin (23, 24). Recently, cross-linking of L1 expressed in heterologous cells has shown to trigger the activation of ERK2, a component of the MAPK signal cascade. ERK2 activation appears to be coupled with L1 internalization and phosphorylation of two cytoplasmic serine residues that are conserved in the L1 subgroup (25).

Although the cytoplasmic tails are the most conserved segments of the L1-type molecules, there are also some differences, particularly at their COOH termini. These differences might provide the structural basis for individual intracellular interactions and therefore distinct functional features within the L1 subgroup. To identify proteins that might mediate sig-

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The abbreviations used are: NgCAM, neuron-glia CAM; FNIII, fibronectin type III; CAM, cell adhesion molecule; NrCAM, NgCAM-related CAM, CHL1, close homologue of L1; pro-TGF-α, protransforming growth factor α; AD, GALA activation domain; BD, GALA DNA-binding domain; ST, syntenin; MBP, maltose-binding protein; GST, glutathione S-transferase; EGFP, enhanced green fluorescence protein; GPI, glycosylphosphatidylinositol; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase(s); PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride.
naling, spatial targeting, or trafficking of L1-type molecules by direct interaction with their cytoplasmic segments, we performed yeast two-hybrid screens of brain cDNA libraries. Here, we demonstrate that syntenin-1 is an intracellular binding partner of neurofascin but not of L1 or NrCAM. Syntenin-1 contains two PDZ domains. PDZ domains are multifunctional protein-binding modules, which were first identified in PSD-95,DlgA, and ZO-1, and are now found in a growing number of other cytoplasmic proteins (26). The PDZ domains of syntenin-1 have been previously shown to interact with the COOH termini of syndecans, class B ephrins, EphA7, pro-TGF-β, neurexins, and the union exchanger AE2 (27–32). In this study, we identified the second PDZ domain of syntenin-1 as a binding site of the COOH terminus of neurofascin and of several other transmembrane proteins mentioned above. Neurofascin was found not to interact with syntenin-2a or -2b, two isoforms of a novel protein closely related to syntenin-1. Furthermore, we observed a homo- and heterodimerization of syntenin-1 and syntenin-2 that appears to involve larger portions of these molecules. This capacity for self-association might be crucial for homo- and heterotypic clustering of neurofascin and other syntenin-binding proteins.

**Experimental Procedures**

cDNA Constructs Used in the Yeast Two-hybrid System—cDNAs encoding cytoplasmic segments of chick neurofascin and NrCAM, both wild-type and mutants, as well as rat L1, human syndecan-3, pro-TGF-β, ephrin-B2, and EphA7, were obtained by PCR using specific primers and inserted in-frame into the pGBT9 vector, pGAD10 vector containing cDNA corresponding to the 435 COOH-terminal amino acid residues of human neurexin I-α was obtained by a yeast two-hybrid screen with syntenin-1 as a bait. cDNAs encoding cytoplasmic segments of neuroginan-1-67 and -150 were subcloned into the pGAD9 vector from PRIT3 plasmids provided generously by M. Hortusi (University of Michigan, Ann Arbor, MI).

The rat syntenin-1 cDNA that was isolated by the yeast two-hybrid screen was subcloned from the library vector pGAD10 into the pGAD9 and pGAD42 (all from CLONTech). cDNA fragments encoding the 115 NH₂-terminal amino acids, the 30 COOH-terminal amino acids, or lacking the 101 NH₂-terminal amino acids of rat syntenin-1 were amplified by PCR using specific primers and inserted in-frame into the pGAD9 vector and pGAD42 vectors. A syntenin-1 construct encoding the NH₂-terminal segment, PDZ1 and the first seven amino acids of PDZ2 was generated from pGAD424(ST-1) by restriction digestion with NsI and PstI and ligation of the overlapping plasmid ends. The cDNA of a mutant lacking 141 NH₂-terminal amino acids, including 29 residues of PstI and NsiI neurexins, and the anion exchanger AE2 (27–32). In this study, subsequently inserted also into the pGBT9 vector.

nin-1, to one strand of the denatured pGAD10(ST-1) plasmid. The selection primer 5’-pCCCTGACTTTCTCGACTTGGT, which car-

The PCR products were inserted in-frame into the pEGF9 and pGAD42 vectors. In addition, for analytical reasons, a downstream primer 5’-GGAATTCATCCGAGGGTGGTTGCCCTTTGCTG. The PCR products and nucleotide substitutions were introduced into the rat syntenin-1 encoding cDNA by simultaneously annealing the mutagenic primers 5’-CAGTGGACATGTTGACTTTATCTTTAAAAGTGG, respectively, and

The PCR products were inserted in-frame into the pGEX-3X (Amersham Pharmacia Biotech) or pMAL-c2 (New England BioLabs) expression vectors, respectively. MBB-γ-galactosidase (α-fragment) fusion protein was expressed using the pMAL-c2 vector. After transformation of Escherichia coli strain M15(pREP4) (Qiagen), recombinant proteins were purified by affinity chromatography using glutathione-Sepharose 4B (Amersham Pharmacia Biotech) or amylose resin (New England BioLabs) columns as recommended by the manufacturer.

**Cell Cultivation and Transfection**—COS7 and L929 cells were cultivated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, penicillin, and streptomycin at 37 °C and 5% CO₂. COS7 cells were transiently transfected by the DEAE-dextran/Me₂SO method as described previously (11). To transiently transfet L929 cells, cells were harvested from 10-cm plates with trypsin, washed twice, and resuspended in 0.4 ml of RPMI with 100 μl dithiothreitol. Electroporation of the cell suspension was performed in a 4-mm cuvette with 12 μg of DNA using an EasyJetT PLUS device (Eurogentec) at 350 V, 600 microfarads, and room temperature. Immediately after electroporation, cells were transferred in a 5-cm PetriPERM dish (hereaus) precoated with 0.5 mg/ml collagen type I (Sigma) and cultivated for 48 h under standard conditions.

**Western Blot Analysis**—To generate EGFP-syntenin-1, -2a, and -2b fusion proteins, syntenin-1 cDNA was inserted in-frame into the pEGFP-C1, syntenin-2a and -2b into the pEGFP-C2 vectors (CLON-TECH). To obtain these proteins without any tags, we removed the EGFP encoding segment from the pEGFP-C1(ST-1) or inserted syntenin-2a and -2b encoding cDNAs into the pSG5 expression vector (Stratagene). COS7 cells were solubilized 48 h after transfection at 4°C in immunoprecipitation buffer (50 mM Tris(HCl), pH 7.4 as indicated) and centrifuged twice for 20 min at 100,000 g and 4 °C. 50 μg of each protein extract were separated on SDS-PAGE followed by electrotransfer to a PVDF membrane (Amersham Pharmacia Biotech). Rabbit anti-GFP antibodies (IgG fraction, CLONTECH) were applied at a dilution of 1:2500. Anti-syntenin-1 antibodies were applied at a dilution of 1:2500. Anti-syntenin-1 antibodies were obtained from the anisalbumin (anti-syntenin-1) antibody was isolated from the serum of rabbits immunized with the COOH-terminal region of the cytoplasmic domain of syntenin-1. After 48-h cultivation, cells were solubilized in radioimmune precipitation buffer containing protease inhibitors and centrifuged. Tissues of 10-week-old rats were homogenized on ice in radioimmune precipitation buffer containing protease inhibitors as described previously (11). To transiently transfet L929 cells, cells were transferred in a 5-cm PetriPERM dish (hereaus) precoated with 0.5 mg/ml collagen type I (Sigma) and cultivated for 48 h under standard conditions.

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RESULTS

Identification of Rat Syntenin-1 as a Neurofascin-binding Protein—To identify proteins that interact with the cytoplasmic segments of the L1-type cell adhesion molecules, we performed several yeast two-hybrid screens. One screen of a rat brain cDNA library using the cytoplasmic segment of chicken neurofascin as a bait (90% identity with rat neurofascin at the level of the amino acid sequence) resulted in the identification of eight positive clones out of 6 \times 10^6 HF7c yeast transformants. The 1.3 kb cDNA inserts isolated from three positive clones were identical and encoded a 432-amino acid protein—coding frame flanked by 15 bp of the 5′- and 1.1-kb-long 3′-uncoding region. Conceptual translation of the open reading frame revealed a sequence of 300 amino acids that shows 91% identity with human syntenin (27) and can be considered to represent the rat homologue of human syntenin. Subsequently, it will be referred to as syntenin-1 (see below) (Fig. 1A). Syntenin-1 is a cytoplasmic protein consisting of a tandem of two PDZ domains flanked by an NH2-terminal segment of 112 amino acid residues and a short COOH-terminal stretch of 26 residues. Both these flanking segments do not show any significant similarities to any known polypeptide modules. Furthermore, on the basis of the GenBank database entries, we cloned two isoforms of rat syntenin, termed syntenin-2 in the following, which are highly related to the syntenin-1 (see below) (Fig. 1A and B). The stop codon containing the 5′-uncoding region of the cloned cDNA of the shorter isoform, designated syntenin-2p, and of the putative β-isoforms of syntenin-1 are indicated in brackets.

7.5% SDS-PAGE and blotting to PVDF membranes (Amersham Pharmacia Biotech). Blots were either stained with corresponding antibodies or incubated overnight at 4 °C with 40 μg/ml MBP-syntenin-1 or MBP-β-galactosidase fusion proteins in PBS, pH 7.4, supplemented with 0.2% bovine serum albumin and 0.05% Tween-20. Ligand binding was visualized using anti-MBP antiserum (1:10,000, New England Biolabs), horseradish peroxidase-conjugated secondary antibodies (1:10,000, Dianova), and a Metal Enhanced DAB substrate kit (Pierce).

Analysis of Colocalization of Neurofascin and Syntenin-1—L929 cells were transiently cotransfected with EGFFP-syntenin-1 and wild-type neurofascin or the truncated GPI-anchored neurofascin construct described above. To induce and visualize clustering of neurofascin, cells were incubated in culture medium with 4% formaldehyde for 10 min, cells were covered with 50% glycerol in PBS and processed for confocal imaging using a Bio-Rad MRC-1000 system.

Surface Plasmon Resonance Analysis—200 RU (resonance units) of synthetic neurofascin peptides corresponding to the last 15 COOH-terminal amino acids of either its wild-type or the A0S-substituted sequence and containing an additional NH2-terminal lysine residue were immobilized in culture medium with 4% formaldehyde for 10 min, cells were covered with 50% glycerol in PBS and processed for confocal imaging using a Bio-Rad MRC-1000 system.

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distinct from that of the longer 2α-isofrom. This finding might be taken as evidence that syntenin-2β is generated by alternative splicing instead of using the methionine residue at position 86 within the sequence of syntenin-2a as an alternative translational start site (data not shown and Fig. 1, A and B).

To study the tissue expression pattern of syntenin-1 and to compare it with neurofascin, we generated antibodies against bacterially expressed GST-syntenin-1 fusion protein in rabbits. To rule out a possible cross-reactivity with syntenin-2, Western blots of syntenin-1, -2α, -2β or EGFP fusion proteins of these expressed in COS7 cells were analyzed by generated antibodies. Syntenin-1 migrated with a molecular mass of ~36 kDa (predicted 32.4 kDa), whereas syntenin-2α (predicted molecular mass 34.4 kDa) was weakly detected as a single band at ~39 kDa, indicating that both proteins can be distinguished on the basis of their apparent molecular masses in SDS-PAGE (Fig. 2A). Although syntenin-1 and -2 were expressed at equal amounts in COS7 cells as judged from the staining intensities of EGFP-syntenin-1 and -2 fusion proteins using antibodies against GFP (Fig. 2B), comparison of the staining intensities demonstrates that the anti-syntenin-1 antibodies reacted only very weakly with syntenin-2α and do not detect syntenin-2β (Fig. 2C). To analyze the expression pattern of syntenin-1 and neurofascin, Western blots of detergent extracts of various rat tissues were performed. In contrast to neurofascin, which was found to be expressed exclusively in the brain (Fig. 2E), syntenin-1 with an apparent molecular mass of ~36 kDa, as also observed in transfected COS7 cells, revealed a wide-spread expression pattern (Fig. 2D). Comparison of the staining intensities indicated strongest expression of syntenin-1 in brain followed by testis, lung, and heart. The lowest level of syntenin-1 staining was detected in skeletal muscles and liver. Syntenin-2 was not detected in this blot, most likely due to the low cross-reactivity of the anti-syntenin-1 antibodies as described above. The observed tissue expression pattern of syntenin-1 suggests that functions of syntenin-1 are not restricted to neurofascin, consistent with former studies on syntenin/TACIP18.

Syntenin-1 but Not Syntenin-2 Binds to the COOH Terminator of Neurofascin—Most PDZ domains investigated so far bind directly to the COOH termini of transmembrane proteins. The specificity of these interactions is determined by the structural features of the respective PDZ binding pockets and the COOH-terminal amino acids of transmembrane molecules (38). We therefore investigated whether syntenin-1 interacts with the COOH terminus of neurofascin (SLA-COOH in chick and rat) and with the COOH termini of other L1 subgroup members. In particular, NrCAM and neuroglian share a class I PDZ binding motif (S/T)(X)(V/I) at their COOH termini. First, we tested different deletion and substitution constructs in the yeast two-hybrid system. Fourteen of the most COOH-terminal amino acids of neurofascin were sufficient to bind syntenin-1, whereas deletion of the alanine residue at the position 0 abolished the interaction completely. This demonstrates that the COOH terminus of neurofascin is the binding site for syntenin-1 (Table 1). Moreover, we showed that the interaction with syntenin-1 is not affected by the alternative splicing of the neurofascin cytoplasmic exon, which encodes the four-amino acid residue stretch RSLE. This sequence motif is also differentially spliced in L1/NgCAM and NrCAM (1). In contrast, syntenin-1 interacted only with the long, nervous system-specific splice isoform (COOH-terminal tripeptide TYV) of the intracellular segment of Drosophila neuroglian but not with its short form (KGL-COOH) that is widely expressed (39). As expected, syntenin-1 failed to interact with the cytoplasmic tail of L1 (ALE-COOH) and, surprisingly, with that of NrCAM (SFV-COOH). Because the fourth vertebrate member of the L1 group CHL1 does not contain any appropriate COOH-terminal PDZ binding motif (LRA-COOH), similarly to L1, its interaction with syntenin-1 was not analyzed. The cloning of syntenin-2α and -2β allowed us also to test whether they bind to any member of the L1 subfamily or to the other transmembrane proteins targets that are known to interact with syntenin-1. Among these, only neurxin I, which shares its COOH-terminal sequence with neur...
interaction, the apparent dissociation rate constant was calculated to be $-1 \times 10^{-4} \text{s}^{-1}$. However, a further analysis of the obtained binding curves revealed that they are not consistent with such a simple model. In contrast, no significant binding was detected using the A(0)S-substituted neurofascin peptide with MBP-syntenin-1, or the wild-type peptide with MBP-β-galactosidase.

To demonstrate the association of syntenin-1 with neurofascin in mammalian cells, L929 cells were cotransfected with plasmids encoding syntenin-1 fused to EGFP and either wild-type neurofascin or the truncated GPI-anchored neurofascin construct (Fig. 3C). Colocalization of neurofascin clustered by antibodies and EGFP-syntenin-1 was observed in a subpopulation of cells expressing wild-type neurofascin but not in the control cultures. This further confirmed the specificity of the investigated interaction.

**Neurofascin and Several Other Transmembrane Proteins (Neuroglian-180, Pro-TGF-α, Syndecans, B-ephrins, EphA7, and Neurexins) Bind to the Second PDZ Domain of Syntenin-1**—Syntenin-1 contains two PDZ domains and interacts with the COOH terminus of neurofascin and several other transmembrane proteins. This suggests that at least one of these domains is responsible for these interactions. To determine the individual binding specificity of the two syntenin-1 PDZ domains for different known interacting transmembrane proteins, we generated several syntenin-1 mutants and tested these in the two-hybrid assay. We observed that neither of the overlapping deletion constructs of syntenin-1, which were composed of the NH2-terminal third only or together with PDZ1, or of the isolated PDZ2 with the COOH-terminal stretch, did interact with the cytoplasmic tail of neurofascin (data not shown). This is consistent with the published data on the interaction of syntenin-1 with syndecan or pro-TGF-α (28, 29).

To address the binding specificity of the syntenin-1 PDZ domains further and to map binding sites within syntenin-1, we substituted the last glycine residue in the carboxylate-binding loop of each PDZ domain (see Fig. 1A) by glutamate (G128E in PDZ1) or aspartate (G212D in PDZ2). This glycine is the most conserved residue throughout all PDZ domains and allows the loop preceding the β2 strand to form a turn that is necessary for interaction with the ligand’s carboxylate group (40). Three resulting point mutants (PDZ1∗, PDZ2∗, and PDZ1∗2∗) were then tested for binding to the cytoplasmic tails of several transmembrane proteins listed in Table II. The mutation in the first domain (PDZ1∗) did not cause any significant reduction of the binding as observed in the two-hybrid assay. In contrast, the PDZ2∗ construct, as well as the double-mutant PDZ1∗2∗, failed to interact with any of the tested cytoplasmic segments. This indicates that the second PDZ domain is required to bind them. However, the deletion construct of syntenin-1 containing the PDZ tandem but lacking 101 NH2-terminal amino acids (NA101) also failed to bind neurofascin, neuroglian-180, and pro-TGF-α, whereas the intensity of its interaction with syndecan-3, ephrin B-2, or EphA7 was reduced (Table II).

Taken together, we conclude that neurofascin and the other tested transmembrane proteins bind to the second syntenin-1 PDZ domain that is inactive if separated from other parts of the molecule. In addition, the specificity of this domain appears to be unusual, in that it is able to bind class I (neurofascin, neuroglian, and pro-TGF-α) and class II (syndecans, class B ephrins, EphA7, and neurexins) COOH termini. Abolishment or reduction of binding caused by the deletion of the PDZ1 and/or the NH2-terminal segment of syntenin-1 might be the result of inappropriate folding of the obtained polypeptides. Alternatively, these domains might be indirectly involved in the interaction between syntenin-1 and the transmembrane proteins. One such conceivable mechanism might include the oligomerization of syntenin-1.

**Homo- and Heterodimerization of Syntenin-1 and Syntenin-2**—To further our understanding of the molecular functions of syntenin-1 and syntenin-2, we examined the ability of these proteins to self- and heteroassociate using the two-hybrid assay. Full-length syntenin-1 as well as syntenin-2α showed a

### Table I

| BD-cyttoplasmic segments | β-Galactosidase |
|--------------------------|----------------|
| Nf-AV (wt)               | +/+            |
| Nf-AS (wt)               | +/+            |
| Nf-AV (mut)              | –              |
| Nf-AS (mut)              | –              |
| Nf-LF (wt)               | +/+            |
| Nf-SA (wt)               | +/+            |
| Nf-YF (wt)               | +/+            |
| Nf-YN (wt)               | +/+            |
| Nf-IY (wt)               | +/+            |
| Nf-IY (mut)              | –              |
| Nf-IY (mut)              | –              |
| Nf-IY (mut)              | –              |
| Nf-IY (mut)              | –              |
| Nf-IY (mut)              | –              |
| Nf-IY (mut)              | –              |
| Nf-IY (mut)              | –              |
| Nf-IY (mut)              | –              |
| Nf-IY (mut)              | –              |
| Nf-IY (mut)              | –              |
| Nf-IY (mut)              | –              |
| Nf-IY (mut)              | –              |
| Nf-IY (mut)              | –              |
| Nf-IY (mut)              | –              |
| Nf-IY (mut)              | –              |
| Nf-IY (mut)              | –              |
| Nf-IY (mut)              | –              |

**Interaction of Neurofascin with Syntenin-1**

Binding of syntenin-1 to the two wild-type isoforms (±RSL6 exon) as well as to the various COOH-terminal point mutants of the cytoplasmic segment of neurofascin (Nf) were analyzed by a yeast two-hybrid assay. The five COOH-terminal amino acid residues are replaced. Mutated residues are printed in boldface. The Nf-c14 construct contains only the last 14 COOH-terminal amino acid residues of neurofascin. In addition, neuroglian (Ngl-167 and Ngl-180 isoforms), L1, NrCAM (Nr), and two mutants of the latter were investigated. In the NrCAM mutant Nr-Nf the five COOH-terminal residues are replaced by those of neurofascin. The results of the β-galactosidase filter assays (the time it takes colonies to start turning blue) were scored as follows: + + + + + 30 min < + + + + 1 h < + + 2 h < + + + 4 h < –.

To define amino acid residues that determine the binding of syntenin-1 to wild-type neurofascin containing the cytoplasmic tail and/or the NH2-terminal segment of syntenin-1 might be the cause further to map binding sites within syntenin-1, we substituted the last glycine residue in the carboxylate-binding loop of each PDZ domain (see Fig. 1A) by glutamate (G128E in PDZ1) or aspartate (G212D in PDZ2). This glycine is the most conserved residue throughout all PDZ domains and allows the loop preceding the β2 strand to form a turn that is necessary for interaction with the ligand’s carboxylate group (40). Three resulting point mutants (PDZ1∗, PDZ2∗, and PDZ1∗2∗) were then tested for binding to the cytoplasmic tails of several transmembrane proteins listed in Table II. The mutation in the first domain (PDZ1∗) did not cause any significant reduction of the binding as observed in the two-hybrid assay. In contrast, the PDZ2∗ construct, as well as the double-mutant PDZ1∗2∗, failed to interact with any of the tested cytoplasmic segments. This indicates that the second PDZ domain is required to bind them. However, the deletion construct of syntenin-1 containing the PDZ tandem but lacking 101 NH2-terminal amino acids (NA101) also failed to bind neurofascin, neuroglian-180, and pro-TGF-α, whereas the intensity of its interaction with syndecan-3, ephrin B-2, or EphA7 was reduced (Table II).

Taken together, we conclude that neurofascin and the other tested transmembrane proteins bind to the second syntenin-1 PDZ domain that is inactive if separated from other parts of the molecule. In addition, the specificity of this domain appears to be unusual, in that it is able to bind class I (neurofascin, neuroglian, and pro-TGF-α) and class II (syndecans, class B ephrins, EphA7, and neurexins) COOH termini. Abolishment or reduction of binding caused by the deletion of the PDZ1 and/or the NH2-terminal segment of syntenin-1 might be the result of inappropriate folding of the obtained polypeptides. Alternatively, these domains might be indirectly involved in the interaction between syntenin-1 and the transmembrane proteins. One such conceivable mechanism might include the oligomerization of syntenin-1.

**Homo- and Heterodimerization of Syntenin-1 and Syntenin-2**—To further our understanding of the molecular functions of syntenin-1 and syntenin-2, we examined the ability of these proteins to self- and heteroassociate using the two-hybrid assay. Full-length syntenin-1 as well as syntenin-2α showed a...
strong homotypic interaction and a weaker heterotypic interaction as judged by the β-galactosidase assay (Tables III and IV). To gather additional evidence for the homotypic oligomerization of syntenin-1, we transiently cotransfected COS7 cells with plasmids encoding Myc- and FLAG-tagged syntenin-1. Specific coprecipitation of Myc- or FLAG-syntenin-1 from detergent lysates of double-transfected cells with either anti-FLAG or anti-Myc monoclonal antibodies, respectively, was readily observed (Fig. 4A). Full-length syntenin-1 without any epitope extensions also coprecipitated with FLAG- as well as with Myc-syntenin-1, suggesting that oligomerization might not be caused by the Myc or FLAG epitopes (data not shown). In addition, size exclusion chromatography of purified bacterially expressed MBP-syntenin-1 fusion protein revealed three peaks. One peak, estimated at a molecular mass of ~90 kDa, fits relatively well with the monomeric form (predicted molecular mass 77 kDa), whereas the other at ~163 kDa represents most likely the dimeric form of recombinant MBP-syntenin-1 (Fig. 4B). A third MBP-syntenin-1 peak was observed in the exclusion volume of the column and probably contains supermolecular aggregates. Taken together, these investigations indicate that syntenin-1 self-associates and forms homodimers.

To address the question of the structural basis of the self- and heteroassociation of syntenin-1 and -2, we again used the yeast two-hybrid system to test different combinations of wild-type, deletion, and point mutants of syntenin-1 as well as the two isoforms of syntenin-2. No interaction of full-length syntenin-1 with overlapping deletion constructs consisting of either the NH2-terminal domain alone or combined with PDZ1, of PDZ2 with the COOH-terminal stretch, or of the COOH-terminal stretch of 30 amino acid residues alone could be detected by the two-hybrid assay (data not shown). In contrast, full-length syntenin-1 bound to the syntenin-1 construct lacking its NH2-terminal third with only slightly reduced intensity, whereas this ΔN101 mutant was not able to self-associate (Table III). These observations indicate that the NH2-terminal third might be required but is not sufficient for the homotypic interaction of syntenin-1 and that it might bind to some part of the molecule other than itself. Similarly, syntenin-2β, which is comparable to the NΔ101 construct of syntenin-1 (see Fig. 1), was found to self-associate significantly weaker than the long α-isofrom (Table IV). We tested whether the point mutations within carboxy-terminal regions of PDZ domains can affect the self-association of syntenin-1. For this reason, PDZ1*, PDZ2*, and PDZ1*2* mutants were assayed for their ability to interact with each other, wild-type, or deletion constructs of syntenin-1. Only results were considered that could be confirmed by a vice versa exchange of the two-hybrid vectors (BD and AD) in which a particular pair of constructs was expressed. The data illustrate an apparent implication of the PDZ domains in the self-association mechanism of syntenin-1, but details remain to be investigated in the future.

Taken together, our observations indicate that the individual domains alone are not sufficient and the overall integrity of syntenin-1 is required for the self-association of syntenin-1. The inhibition of the homodimerization of syntenin-1 might concomitantly affect its PDZ-mediated binding to neurofascin and to other tested transmembrane proteins.

**DISCUSSION**

In this study, we identified the PDZ domains containing molecule syntenin-1 as an intracellular neurofascin binding partner. The interaction of syntenin-1 with the cytoplasmic domain of neurofascin was observed in the yeast two-hybrid system and confirmed by overlay assay, by surface plasmon resonance measurements, and by the colocalization of both proteins in transfected cells. In addition to rat syntenin-1, we isolated a novel human syntenin-1-related molecule, syntenin-2, which can be expressed as a long isoform (syntenin-2α) that has the same domain organization as syntenin-1 and as a short isoform (syntenin-2β) that contains the PDZ-tandem but lacks mostly the NH2-terminal third. Syntenin-2α but not syntenin-2β was shown to interact with neurexins, but neither with neurofascin nor with several other transmembrane proteins. Syntenin-1 and syntenin-2 both were able to self-associate and to interact with each other in the two-hybrid system. The homodimerization of syntenin-1 was confirmed by coimmunoprecipitation experiments and gel filtration chromatography. Although the binding sites sufficient for the homodimerization of syntenin-1 are still unknown, we consider a homotypic binding mode, in which the PDZ tandem and at least a part of the NH2-terminal domain are essential. The binding of the syntenin-1 deletion mutant NΔ101 to wild-type syntenin-1 and its failure to interact with itself exclude the possibility that dimers are formed by association of the NH2 termini of two syntenin-1 molecules. More likely, binding of the NH2 terminus of syntenin-1 to an unknown site within the COOH-terminal two thirds of the molecule enables an antiparallel or “head to tail” association.

We demonstrated that other vertebrate members of the L1 subgroup of cell adhesion molecules bind neither syntenin-1 nor syntenin-2. Interestingly, syntenin-1 was also able to interact in yeast with the nervous system-specific isoform of the cytoplasmic tail of *Drosophila* L1-type protein neuroglian. This

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**Table II**

| Proteins | Neurofascin | Syntenin-1 | 1/WT | 1/ΔN101 | 1/PDZ1* | 1/PDZ2* | 1/PDZ1*2* | 2α/WT | 2β/WT |
|----------|-------------|------------|------|--------|--------|--------|--------|--------|--------|
| Neurofascin | -YSLACOOH | Neuroglian180 | + + + + | - | + + + | - | - | - | - |
| Neuroglian180 | -ATYVCOOH | Pro-TGF-α | + | + | + | + | - | - | - |
| Pro-TGF-α | -ETVVC OOH | Syntenin-1 | + | + | + | + | - | - | - |
| Syntenin-1 | -EFYA COOH | Ephrin-B2 | + + + + | + + + + | + + + + | - | - | - | - |
| Ephrin-B2 | -YKVCOOH | EphA7 | + + + + | + + + + | + + + + | - | - | - | - |
| EphA7 | -GIQV COOH | Neurexin 1 | + + + + | + + + + | + + + + | - | - | - | - |
| Neurexin 1 | -EYYV COOH | - | + + + + | + + + + | + + + + | - | - | - | - |

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**Figure 4**

In this study, we identified the PDZ domains containing molecule syntenin-1 as an intracellular neurofascin binding partner. The interaction of syntenin-1 with the cytoplasmic domain of neurofascin was observed in the yeast two-hybrid system and confirmed by overlay assay, by surface plasmon resonance measurements, and by the colocalization of both proteins in transfected cells. In addition to rat syntenin-1, we isolated a novel human syntenin-1-related molecule, syntenin-2, which can be expressed as a long isoform (syntenin-2α) that has the same domain organization as syntenin-1 and as a short isoform (syntenin-2β) that contains the PDZ-tandem but lacks mostly the NH2-terminal third. Syntenin-2α but not syntenin-2β was shown to interact with neurexins, but neither with neurofascin nor with several other transmembrane proteins. Syntenin-1 and syntenin-2 both were able to self-associate and to interact with each other in the two-hybrid system. The homodimerization of syntenin-1 was confirmed by coimmunoprecipitation experiments and gel filtration chromatography. Although the binding sites sufficient for the homodimerization of syntenin-1 are still unknown, we consider a homotypic binding mode, in which the PDZ tandem and at least a part of the NH2-terminal domain are essential. The binding of the syntenin-1 deletion mutant NΔ101 to wild-type syntenin-1 and its failure to interact with itself exclude the possibility that dimers are formed by association of the NH2 termini of two syntenin-1 molecules. More likely, binding of the NH2 terminus of syntenin-1 to an unknown site within the COOH-terminal two thirds of the molecule enables an antiparallel or “head to tail” association.

We demonstrated that other vertebrate members of the L1 subgroup of cell adhesion molecules bind neither syntenin-1 nor syntenin-2. Interestingly, syntenin-1 was also able to interact in yeast with the nervous system-specific isoform of the cytoplasmic tail of *Drosophila* L1-type protein neuroglian. This
finding raises the question whether there is a syntenin-1-like molecule in flies. Because neuroglian has been considered as the sole L1-type molecule in *Drosophila* (3), it might be able to substitute for several functions that are conferred by diverse L1-type molecules (neurofascin, NrCAM, L1, CHL1) in vertebrates, including binding to a putative *Drosophila* syntenin-1-related protein. Although we failed to identify a syntenin-1-like protein in the complete *Drosophila* genomic data base, another PDZ protein might interact with the long neuroglian isoform in the insect cells.

Although the majority of known PDZ domains bind to specific COOH-terminal peptides of transmembrane molecules, several of them interact with internal sequences or with other PDZ domains. Here, using site-directed mutagenesis, we mapped the binding sites to the COOH terminus of neurofascin and to the PDZ2 domain of syntenin-1. Moreover, we found that PDZ2 is also responsible for the interaction with neuroglian-180 and several other transmembrane proteins that were previously reported to bind syntenin-1. One of them is pro-TGF-α, which, together with neurofascin and neuroglian-180, contains a threonine or a serine at the COOH-terminal position –2 and belongs therefore to the class I PDZ-binding proteins (38). Another group of syntenin-1-interacting proteins tested here consists of syndecans, neurexins I–III, class B ephrins, and EphA7, all of which belong to the class II PDZ-binding proteins that contain an aromatic or hydrophobic residue at position –2. On the basis of their sequences, the PDZ domains of syntenin-1 should interact with the class II-specific sequence motifs (28). Indeed, affinity of syntenin-1 to class II COOH termini appears to be higher than to class I COOH termini. Nevertheless, our data together with the observations of Fernandez-Larrea et al. (29) on pro-TGF-α strongly support that the syntenin-1 PDZ2 domain also binds to specific class I COOH termini. Screens of oriented peptide libraries and yeast two-hybrid studies have demonstrated that PDZ binding may require side-chain interactions in addition to those at the COOH-terminal amino acid positions 0 and –2 (38, 41). Here, we showed that binding to syntenin-1 PDZ2 domain is also determined by the –3 residue of the ligand. NrCAM containing the COOH-terminal PDZ-binding motif SXV-COOH and an asparagine residue at position –3 failed to bind syntenin-1, whereas the single substitution Yt–3N within the sequence of neurofascin abolished its interaction with syntenin-1 in yeast. Moreover, the reciprocal substitution Nt–3Y within the COOH-terminal sequence of the NrCAM enabled this mutant to bind. These findings, however, leave open the possibility for other critical positions in addition to 0, –1, and –3, as well as the question of the structural basis of the unusual ligand recognition by the syn-
neurofascin at nodes of Ranvier in the optic nerve of adult mouse,\(^3\) making a scaffolding function of syntenin-1 at least at this site less likely. On the other hand, homo- and heterodimerization of syntenin-1 may allow a large spectrum of transmembrane proteins to subdomains of the plasma membrane of neural cells. However, syntenin-1 was not found to co-cluster with neurofascin at nodes of Ranvier in the optic nerve of adult mouse,\(^3\) making a scaffolding function of syntenin-1 at least at this site less likely. On the other hand, homo- and heterodimerization of syntenin-1 may allow a large spectrum of transmembrane receptors belonging to different protein families to be assembled together with neurofascin.

Neurofascin has been shown to be implicated in axonal growth and fasciation (10, 11, 35). These dynamic cellular processes require a mechanism to regulate the cell surface expression of neurofascin. One possible way to modulate the number of neurofascin molecules on the neural surface might be to internalize or to target it to specific cell surface domains. Insertion and removal of plasma membrane components are part of the growth cone machinery, and evidences have been accumulated in the past that vesicular transport and the subcellular targeting of proteins in neurons are involved in neurite extension (45). Because syntenin-1 appears to be necessary for correct targeting of pro-TGF-\(\alpha\) to the surface of Chinese hamster ovary cells (29) and is colocalized with internalized trans-

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\(^3\) M. Koroll and F. G. Rathjen, unpublished observations.
ferrin in early apical recycling endosomes in Madin-Darby canine kidney cells (46), it might function by linking bound neurofascin or other transmembrane proteins to trafficking or recycling pathways also in neural cells. The identification of syntenin-1 as an intracellular binding partner of neurofascin may allow us to study the removal and insertion of neurofascin in the context of neurite extension during early neural development or its targeting to axonal initial segments and to the nodes of Ranvier in the differentiated nervous system. Further insights into the functions of syntenin-1 and -2 might also be obtained by identifying cytoplasmic proteins linking them to trafficking or signaling pathways.

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The Neural Cell Recognition Molecule Neurofascin Interacts with Syntenin-1 but Not with Syntenin-2, Both of Which Reveal Self-associating Activity
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