Syntenin-Syndecan Binding Requires Syndecan-Synteny
and the Co-operation of Both PDZ Domains of Syntenin*

Syntenin is an adaptor-like molecule that binds to the cytoplasmic domains of all four vertebrate syndecans. Syntenin-syndecan binding involves the C-terminal part of syntenin that contains a tandem of PDZ domains. Here we provide evidence that each PDZ domain of syntenin can interact with a syndecan. Isolated or combined mutations of the carboxylate binding lysines in the inter-βAβB loops and of the αB1 residues in either one or both the PDZ domains of syntenin all reduce syntenin-syndecan binding in yeast two-hybrid, blot-overlay, and surface plasmon resonance assays. PDZ2 mutations have more pronounced effects on binding than PDZ1 mutations, but complete abrogation of syntenin-syndecan binding requires the combination of both the lysine and the αB1 mutations in both the PDZ domains of syntenin. Isothermal calorimetric titration of syntenin with syndecan peptide reveals the presence of two binding sites in syntenin. Yet, unlike a tandem of two PDZ2 domains and a reconstituted PDZ1+PDZ2 tandem, a tandem of two PDZ1 domains and isolated PDZ1 or PDZ2 domains do not interact with syndecan bait. We conclude to a co-operative binding mode whereby neither of these two PDZ domains is sufficient by itself but where PDZ2 functions as a “major” or “high affinity” syndecan binding domain, and PDZ1 functions as an “accessory” or “low affinity” syndecan binding domain. Pairing, but not the isolated PDZ domains of syntenin bind also strongly to the immobilized cytoplasmic domains of Neurexin and B-class ephrins. The syntenin of compatible bait may result from the assemblies and co-assemblies of syntenins and other similarly suited partners in larger supramolecular complexes. In general, an intramolecular combination of PDZ domains that are weak, taken individually, would appear to be designed to detect rather than drive the formation of specific molecular assemblies.

Syndecans are type-I membrane proteins that are substituted with heparan sulfate. They function as versatile co-receptors, as there is a growing list of examples where the heparan sulfate chains of these membrane proteins are involved in the docking of heparin binding molecules (e.g. growth factors and adhesion molecules) to cell surfaces and facilitate the interactions of these molecules with specific cognate signaling receptors (1). The strict evolutionary conservation of the structures of the cytoplasmic domains of the syndecans implies that the biological functions of these membrane proteins may also depend on highly specific cytoplasmic interactions and associations. Recently, the cytoplasmic domains of the syndecans were shown to interact with syntenin (2) and CASK (3, 4), two proteins that belong to the larger family of proteins that contain one or several PDZ domains (PDZ proteins).

PDZ domains are structural motifs of about 80 amino acids that were initially found in the post synaptic density-95, disc-large, and zonulin-1 proteins but occur in a large variety of proteins (5). PDZ domains interact with the C termini of specific peptide structures. X-ray structures (6, 7) and NMR data (8) show that PDZ domains are compact α + β modules containing five to six β strands (labeled βA to βE) and two α helices (αA and αB). The binding peptide fits into a hydrophobic pocket created by the principal α-helix (αB), the second β-strand (βB), and the “carboxylate binding” loop that connects the βA and βB strands. The terminal carboxylate of the peptide interacts with a cradle of amide nitrogens from the inter-βAβB loop, but an arginine or lysine at the start of this loop also contributes to the stabilization of this carboxylate via a bound water molecule. The C-terminal four residues of the peptide are stabilized by main chain hydrogen bonds with βB, whereby peptide binding represents the augmentation of the PDZ β-sheet by an antiparallel strand. PDZ domains are selective. This binding specificity is achieved by domain interactions with the residue at the −2 position of the bound peptide. Type-I PDZ domains bind to peptides with the terminal-(ST)XV consensus motif, whereby the Ser or Thr hydrogen bonds a relatively well conserved histidine residue near the start of the αB helix (7). Type-II PDZ domains, in contrast, bind to peptides with hydrophobic or aromatic amino acids in the −2 position and feature a non-basic residue as the first residue of the αB helix (9). Most often a PDZ domain occurs in association with other functional protein-protein interaction modules, including other PDZ domains (5). The specific associations and most often multiple binding interactions of PDZ proteins implicate many of these proteins in the localization of receptors and cytosolic effectors to specific membrane sites and in linking extracellular signals to the cytoskeleton and intracellular signaling pathways.

Syntenin contains two PDZ domains, which occur as a direct repeat and compose the C-terminal two-thirds of the protein. Conforming to a PDZ-mediated interaction, the interaction of syntenin with syndecans depends on the integrity of the C-
terminal FYA sequence that is common to all syndecans and qualifies as a class II PDZ-interacting peptide (10). Yet, the syndecan cytoplasmic domain in solution does not bind syntenin or not as strongly as syntenin binds to immobilized syndecans, and the only part of syntenin that appears dispensable for the interaction is the N-terminal domain. This suggests that both PDZ domains might be needed in a two-pronged (socket-plug) attachment of syntenin to di- or oligomerized syndecans (2). Syntenin is not unique in this respect. In GRIP, which contains two clusters of three adjacent PDZ domains, the minimal region required for binding to the AMPA receptor spans two complete PDZ domains (11), and also in the case of the human Dlg protein, two adjacent PDZ domains are needed for interaction with protein 4.1 (12). On the other hand, there are many more examples of PDZ domains that do work in isolation, and several proteins with single PDZ domains have been identified. CASK, originally identified as a membrane-associated guanylate kinase homolog that binds to the t-YVV sequence of neurexin (13) and recently shown to also bind to syndecans (3, 4), is one of these proteins with single PDZ domains that work in isolation. This singles out the PDZ domains of syntenin and several other proteins as peculiar or suggests trivial explanations for this need for paired domains. Conceivably, incorrect folding of PDZ domains that were isolated from their structural contexts may complicate binding assignments. Moreover, some PDZ domains have been shown to form dimers with other PDZ domains (14, 15), further complicating the identification of the peptide-binding sites in those instances where binding would be based on co-therapeutic PDZ domain interactions.

To resolve this issue for the interaction between syntenin and the syndecan cytoplasmic domain, we have introduced point mutations in the two PDZ domains of syntenin, separately or in combination, using the crystal structures of peptide bound to PDZ3 of human Dlg and PSD-95 as a guide. The mutants were used as prey for syndecan bait in yeast two-hybrid reporter assays and expressed as glutathione S-transferase (GST) fusion proteins for use in blotting and surface plasmon resonance experiments. Collectively, the results indicate that both PDZ domains of syntenin bind syndecans, but with unequal strengths, and that only their cooperative binding results in stable syntenin-syndecan interactions. Syntenin does not discriminate between the various syndecans. Syntenin binds also to neurexins and B-class ephrins, with a similar result in stable syntenin-syndecan interactions. Syntenin with unequal strengths, and that only their cooperative binding results in stable syntenin-syndecan interactions. Syntenin mutants were constructed as non-amyloid-β-proteins in pGEX-5x-2. All constructs were verified by DNA sequencing.

Escherichia coli BL21 or ER2566 cells were used as host cells to express the GST fusion proteins. Expression was induced by adding 0.4 mm isopropyl β-D-thiogalactoside to the medium when the culture reached an A500 of 0.6. After induction, the cultures were allowed to grow for another 3 h at 30 °C. Induced cells were collected by centrifugation for 15 min at 8000 × g and 4 °C. All the medium was carefully removed, and the pellet was resuspended in 8 ml of TBS-Tween (100 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, pH 8.0). Aprotinin (3 μg/ml), benzamidine (5 mM), leupeptin (20 μg/ml), pepstatin (3 μg/ml), 6-aminohexanoic acid (5 μg/ml), and phenylmethylsulfonyl fluoride (200 μg/ml) were added as protease inhibitors. The cell suspension was incubated with 2.5 mg of lysozyme for 1 h on ice. After 1 h, the solution was centrifuged for 20 min at 18,000 × g at 4 °C. Except for GST-PDZ2 and GST-PDZ2 Δ PDZ2, which proved insoluble, all the GST fusion proteins were mainly found in the water-soluble phase. This phase was applied to a glutathione-Sepharose 4B column (Amersham Pharmacia Biotech). After washing the column with phosphate-buffered saline, the fusion protein was eluted from the column with reduced glutathione.

**Surface Plasmon Resonance Measurements**—Surface plasmon resonance was measured using a BIAcore 2000 instrument. A total of 900 resonance units of biotinylated synthetic peptide, corresponding to the 32 amino acids that compose the cytoplasmic domain of syndecan-2, was coupled to a streptavidin sensor chip. Analytes (GST fusion proteins) were perfused (10 μl/min) over the chip and capture (Fc2) surfaces in running buffer (0.1 M NaCl, 0.005% surfactant P20, 0.01 M Hepes, pH 7.4). Binding was measured as the difference between the Fc2 and Fc1 binding curves. All bindings were tested at different concentrations of analyte.

**Blotting Assays**—Purified GST fusion proteins or isopropyl β-D-thiogalactoside-induced BL21/E. coli cells expressing these fusion proteins were re-suspended in SDS sample buffer and boiled for 5 min. The samples were cleared by centrifugation and fractionated in 12% SDS-polyacrylamide gel electrophoresis, each lane containing 0.1 μg of fusion protein. Cultured human MCF-7 cells were cultured in phosphate-buffered saline and treated with heparin before fractionation by 12% SDS-PAGE. After electro-transfer to Hybond-C super-membranes, the blots were blocked with 5% nonfat milk powder in TBS-Tween (150 mM NaCl, 0.1% Tween 20, 20 mM Tris-HCl, pH 7.6) and incubated with fusion proteins of GST-myct-syntenin, GST-myct deletion mutants, or GST-syntenin point mutants (10 μg/ml in TBS-Tween). After a 3-h incubation at room temperature, the blots were washed with TBS-Tween and incubated for 2 h at room temperature with anti-myc monoclonal antibody 9E10 or with anti-syntenin mAb 18S, both diluted to 1 μg/ml in TBS-Tween. After rinsing with TBS-Tween, bound anti-myc and anti-syntenin were visualized with peroxidase substrate 4-chloro-1-naphthol (Bio-Rad 1/10,000) and ECL Western blotting detection reagent (Amersham Pharmacia Biotech).

**Yeast Two-hybrid Assays**—The bait, consisting of the cytoplasmic domain of syndecan-2 fused in-frame to the DNA binding domain of Gal4, was constructed by PCR using the BamHI restriction sites of the pAS2 vector (CLONTECH Laboratories). The centrifin, mutant syntenins, and PDZ tandem prey were cloned, respectively, in pGAD10, pGAD424, and pACT2 (all from CLONTECH Laboratories). The pAS2 plasmids were transfected in Y187 yeast cells, whereas the pGAD/pACT plasmids were transfected in CG1945 yeast cells. Diploid cells resulted from mating Y187 with 108 yeast cells. The mating was scored by plating the yeast on double plates minus Leu+ Trp+ (Leu− Trp−). Assayed were for galactose-sensitive mutants. A β-galactosidase activity assay was performed for growth on triple minus plates (Leu− , Trp−, His−) with and without 5 mM 3-aminotriazol. For the βa-βb-loop and aβ mutants only, the β-galactosidase assay was performed for growth on triple minus plates (Leu−, Trp+, His−) with and without 5 mM 3-aminotriazol. For the ATPase activity assay, the results of the growth assay are represented in tabular form in Fig. 4.

**Taq-free Recombinant Syntenin**—Recombinant syntenin, free of any peptide extensions, but with the C-terminal valine substituted by an αB-galactosidase extended by an S-transferase tag was constructed by annealing 5′-GATCCCGAGAATACTGTC- AGAAGAGGATCTGGG to 5′-GATCCCGAATCTCTCCTGAGATGA- GTTTTGGTCCGA and inserting the annealed oligonucleotides into the BamHI site of pGEX-5x-2. Vectors encoding fusion proteins between GST-myc and wild-type syntenin or paired syntenin-PDZ domains were confirmed by generating the corresponding cDNAs in the BamHI site of this modifed vector. Syntenin mutants were constructed as non-amyloid-β-proteins in pGEX-5x-2. All constructs were verified by DNA sequencing.
alanine, was produced in the IMPACT (intein-mediated purification with an affinity chitin binding Tag) T7 system, according to the protocols provided by the supplier (New England Biolabs Inc., Beverly, MA). CDNA-encoding syntenin was produced by PCR using the primers 5'-GGAATTCCATATGTCTCTCTATCCATCTCTCGAAG and 5'-GGCCGCTCTTCCGCAAGCCTCAGGAATGGTGTGGTCCA and cloned in the NdeI and SapI restriction sites of the pTYB1 vector to produce an in-frame fusion between the C terminus of syntenin and the N terminus of an intein-chitin binding domain fusion protein. After propagation of the plasmid in ER2566 E. coli cells and induction of the cells at an A550 of 0.5 with 0.3 mM isopropyl β-D-thiogalactoside at 30 °C for 4 h, the tripartite fusion protein was isolated from crude cell extracts by affinity purification on a chitin column. After extensive washings, the column was treated overnight at 4 °C with 30 mM 1,4-dithiothreitol to induce the intein-mediated self-cleavage of the fusion protein and the consequent release of its syntenin moiety from the column. SDS-PAGE and Coomassie staining revealed only a single peptide band of 33 kDa in the eluate of the DTT-treated column. Alanine was substituted for the C-terminal valine in syntenin because we noted that this substitution markedly increased the efficiency of the excision process and yield of syntenin. The yield was approximately 1 mg/liter of induced culture.

Isothermal Titration Calorimetry—The syntenin solution was dialyzed overnight at 4 °C against 0.1 M NaCl, 0.1 mM EDTA, 20 mM Hepes, pH 8.0 (ITC buffer) before the isothermal titration calorimetry experiment. The titration was performed using an Omega isothermal titration calorimeter from MicroCal™ Inc. (Northampton, MA). The concentration in the sample cell with a volume of 1.33 ml was 0.0980 mM (3.18 mg/ml). The titrated peptide was dissolved to a concentration of 1.72 mM (6.35 mg/ml) in the ITC buffer and injected in fractions of 18 μl. The temperature of the cell was 25 °C. The obtained titration thermogram was analyzed with MicroCal™Origin™ 5.0 software.

RESULTS

βA-βB Carboxylate Binding Loop Mutations—The loop between the βA and βB strands of a PDZ domain is designated as the carboxylate binding loop. Crystal structures indicate that the main chain amides of this loop donate hydrogen bonds to the carboxylate of the terminal (0) residue of the interacting peptide (7). Recognition of this terminal carboxylate also involves the side chain of a basic residue, Arg or Lys, which invariably occurs as the first residue of the loop. In syntenin these basic residues correspond to Lys-119 (PDZ1) and Lys-203 (PDZ2). Speculating that either one or both of these residues would be critical for optimal binding, we replaced these lysines by alanines in either PDZ1 or PDZ2 or in both PDZ1 and PDZ2 and tested the influence of these mutations on the syntenin-s Syndecan interaction. The exact positions of these mutations are shown in Fig. 1.

In yeast two-hybrid assays, syndecan bait only interacts with syntenin prey that contains the two PDZ domains of syntenin. Moreover, in this particular case bait and prey can not be interchanged (2). In this assay, as assessed from colony outgrowth and β-galactosidase staining and using syndecan-2 as bait, none of the Lys3Ala mutations markedly influenced the syndecan-syntenin interaction (Fig. 2B). In surface plasmon resonance (BIAcore) experiments, GST fusion proteins that contain the two PDZ domains of syntenin bind avidly to a syndecan-2 cytoplasmic domain peptide that is immobilized on the sensor chip but not inversely. Moreover, peptide in solution does not compete for syntenin binding to immobilized peptide (2). Binding curves obtained from the perfusions of similar amounts and concentrations of the different GST-syntenin fusion proteins indicated a decreased binding to immobilized syndecan-2 peptide, both for the K119A mutant (PDZ1) and for the K203A mutant (PDZ2) (Fig. 2E). Compared with wild-type
syntenin, the binding of the K203A mutant (PDZ2) was more markedly reduced than that of the K119A mutant (PDZ1). A similarly significant reduced binding was also observed for the K119A/K203A double mutant. A GST-syntenin fusion protein binds also to GST-syndecan-2 fusion proteins that have been fractionated by SDS-PAGE and blotted on nitrocellulose membranes (2). When used as probes in blotting assays, at similar concentrations of GST-syntenin in solution and for similar amounts of GST-syndecan immobilized on the membranes (as assessed by parallel staining with anti-GST), all three Lys [arrow] Ala mutants bound still to wild-type syndecan-2 (Fig. 3). The K203A mutant (PDZ2) and the K119A/K203A double mutant produced signals of almost similar intensities, both clearly lower than the signal obtained with the K119A mutant (PDZ1). Like wild-type syntenin, none of these Lys [arrow] Ala mutants bound to GST-F(C30)S syndecan-2 (substitution of serine for the phenylalanine at the -2 position of the syndecan peptide), consistent with a PDZ-mediated interaction and an unchanged peptide specificity of this interaction (Fig. 3). All together, the negative effects of the lysine to alanine mutations suggested that both PDZ domains were (directly or indirectly) involved in the syndecan binding, possibly with a larger contribution of PDZ2 than of PDZ1.

oB Helix Mutations—The crystal structures of the PDZ domains of PSD95 and human Dlg (see above) also reveal an important role for the first residue of the second α helix of the

![Figure 2. All βA-βB loop mutations and αB helix mutations negatively affect the syntenin-syndecan interaction. Evidence obtained from yeast two-hybrid (A–D) and surface plasmon resonance (E–G) assays. In the two-hybrid system, wild-type (WT) full-length syntenin and the various types of mutant full-length syntenin prey (fused to the activation domain of Gal4) were tested for interaction with the DNA binding domain (BD) of Gal4 and with syndecan-1 (synd1) and syndecan-2 (synd2) bait fused to this Gal4 domain. Activation of the β-galactosidase reporter gene is shown by the blue staining. In the BIAcore experiments, wild-type full-length syntenin (WT) and the various mutant full-length syntenins were expressed as GST-syntenin fusion proteins and tested for binding to the wild-type syndecan-2 cytoplasmic domain peptide. RU, relative units.](image)

![Figure 3. Ligand overlays, testing for the binding of GST-syntenin and GST-syntenin mutants to blotted GST-wild-type syndecan-2 cytoplasmic domain, and GST-F(C30)S mutant syndecan-2 cytoplasmic domain fusion proteins. All GST-syntenin fusion proteins were added at similar concentrations (10 µg/ml overlay solution). Bound syntenin or syntenin mutants were detected using the antisyntenin mAb ICS at 1 µg/ml. Each individual panel represents signals obtained for lanes derived from the same blots, each lane containing a similar amount of wild-type (WT) or mutant (FS) GST-cytoplasmic domain fusion protein. Because of potential variations in transfer efficiencies, signals obtained for different blots (different frames) cannot be directly compared.](image)
domain (the αB1 residue). This αB1 residue interacts with the residue at the −2 position of the bound peptide and determines at least in part the specificity of the domain for the peptide. In the PDZ domains of PSD95 and human Dlg the αB1 residues are histidines that interact with threonines or serines at the −2 position of the bound peptides. The −2 residue in the syndecan peptide is a phenylalanine (t-FYA), and mutating this phenylalanine to alanine indeed abolishes the syndecan-syntenin interaction in yeast two-hybrid and overlay experiments (2). Sequence alignments predict that in syntenin the αB1 residues of the PDZ1 and PDZ2 domains correspond to serine 171 and aspartate 251, respectively. In attempts to alter the binding specificities of the syntenin PDZ domains and reduce their interactions with wild-type syndecans, we converted their αB1 residues into histidines and embedded these in the sequence SHEQ, mimicking the sequence context for the αB1 histidines in PSD95 and human Dlg. The exact positions of these mutations (SDK → HEQ in PDZ1 and KDS → SHE in PDZ2) are represented in Fig. 1. As a complementary test, we also tested whether these mutant PDZ domains would interact with a mutant F(C30)S syndecan-2 cytoplasmic domain, expected to complement for the altered PDZ domain specificity by featuring a serine instead of a phenylalanine at the −2 position.

In the two-hybrid assay with the wild-type syndecan-2 bait, the mutation of the PDZ1-αB helix showed no measurable effect, whereas the mutation of the PDZ2-αB helix (as single mutation or in combination with the PDZ1-αB mutation) abolished the interaction (Fig. 2C). Substituting F(C30)S syndecan-2 for wild-type syndecan-2 as bait in these assays did not support an interaction with wild-type syntenin or the PDZ1αB mutant, but it restored the interaction with the PDZ2αB mutant and the PDZ1αB/PDZ2αB double mutant (not shown). In the BiACore assay (Fig. 2F) using an immobilized wild-type syndecan-2 cytoplasmic domain, the isolated mutation of the PDZ1-αB helix resulted only in a slight decrease in syntenin binding. The isolated mutation of the PDZ2-αB helix and the combination of the αB helix mutations in both PDZ1 and PDZ2 had negative effects. Similar results were obtained in blotting assays, where both the PDZ2αB mutant and the PDZ1αB/PDZ2αB mutant clearly showed reduced binding to wild-type GST-syntenin-2 (Fig. 3). Moreover, unlike wild-type syntenin and the PDZ1αB mutant, the PDZ2αB mutant and the PDZ1αB/PDZ2αB mutant were both effective at binding to the F(C30)S syndecan-2 mutant. All together the results obtained with the αB helix mutants also suggested that both PDZ domains of syntenin were involved in the syntenin-syndecan interaction, but clearly with a larger or more specific contribution of the PDZ2 domain.

**Combined Mutations of the αA-αB Carboxylate Binding Loops and αB Helices**—The combination of the Lys → Ala mutations in the carboxylate binding loops and of the αB helix mutations (KaB mutations) in either PDZ1 or PDZ2 or in both PDZ1 and PDZ2 yielded results that were consistent with the above interpretation. In the two-hybrid assay with the wild-type bait, the K119A/PDZ1αB mutant (KaB mutation of PDZ1) still interacted with syndecan (Fig. 2D). As could have been expected from the effects of the PDZ2αB mutations (see above), the KaB mutation of PDZ2 (K203A/PDZ2αB) or of both PDZ1 and PDZ2 (K119A/PDZ1αB + K203A/PDZ2αB) suppressed this interaction. In the BiACore assay, the KaB mutation of either PDZ1 or PDZ2 had a negative effect on the binding, but the mutation of PDZ2 had a much stronger negative effect than the mutation of PDZ1 (Fig. 2G). Yet only the combination of the KaB mutations of both PDZ1 and PDZ2 completely abolished binding to the syndecan cytoplasmic domain. A similar result was obtained in the blotting assay, where the KaB mutation of PDZ2 strongly reduced binding and the double KaB mutant failed to show any binding to wild-type syndecan-2. None of the KaB mutants bound to F(C30)S mutant syndecan-2 (Fig. 3).

**PDZ Domain Shuffling**—All the above results suggest that the two PDZ domains of syntenin, together, are both involved in the syndecan interaction, yet with unequal contributions. However, such tentative conclusion assumes that the different mutations affect the two domains to a similar extent and affect similar binding mechanisms. We explored this further by constructing fusion proteins with artificial PDZ tandems (Fig. 4A) and comparing these to the PDZ1-PDZ2 tandem in syntenin.

Unlike the PDZ1+PDZ2 and PDZ2+PDZ2 tandems, the PDZ1+PDZ2 tandem failed to interact with the syndecan-2 bait in yeast two-hybrid assays (summarized in Fig. 4B). In the BiACore assay (Fig. 4C), the GST fusion protein with the PDZ1+PDZ2 tandem bound to the syndecan-2 peptide. The extent of this binding approximated that measured for wild-type syntenin. Yet, the shape of the binding curve was different, suggesting much faster association and dissociation kinetics. The GST fusion protein with the PDZ1+PDZ1 tandem, in contrast, bound poorly. Unfortunately, a soluble GST fusion protein with a single PDZ2 or with the PDZ2+PDZ2 tandem could not be obtained. A consistent result was obtained in the blotting assay, where the GST fusion protein with the PDZ1+PDZ2 tandem, but not that with the PDZ1+PDZ1 tandem, bound to the syndecan-2 fusion protein (Fig. 4D). As predicted from prior results (2), GST fusion proteins containing only one copy of PDZ1 did not bind to syndecan in this assay (Fig. 4D).

**Isothermal Titration Calorimetry**—Since the above results suggested syntenin binds to immobilized syndecans using two PDZ domains but not necessarily by both its PDZ domains, we attempted to obtain independent evidence for the number of syndecan-binding sites in syntenin. For that purpose, syntenin was expressed as a syndenin-intein-chitin binding domain fusion protein and recovered as a tag-free protein after intein-mediated self-cleavage of the chitin-bound fusion protein. Like the GST-syntenin fusion protein, this tag-free syntenin bound to a syndecan-2 peptide that was immobilized on sensor chips (Fig. 4C). The tag-free fusion protein was then used for isothermal titration calorimetry experiments. Repetitive injections of the syndecan-2 cytoplasmic domain peptide in a cell filled with syntenin yielded only very low binding heat values, but a binding isotherm with a breakpoint at molar ratio 2, consistent with a model that supposes two peptide-binding sites in syntenin (Fig. 5).

**Alternative Bait for Syntenin**—To test whether a two-PDZ domain requirement also applied to syndecan bait other than syndecan-2, similar yeast two-hybrid and blotting experiments were performed with GAL4 and GST fusion proteins that contained the cytoplasmic domains of the syndecans -1, -3, and -4. In yeast two-hybrid assays, all syndecans acted as bait for syntenin or the paired PDZ domains of syntenin (not shown). All syndecans showed a similar requirement for an integer PDZ2-αB helix in syntenin (as shown for syndecan-1 and -2 in Fig. 2). As a GST fusion protein, syntenin bound equally well to the four different syndecans in blotting experiments (Fig. 6), in contrast to CASK-PDZ, which seems to bind preferentially to syndecan-2 and -4. Thus, unlike syntenin, CASK can interact with syndecans, engaging only a single PDZ domain, and discriminates to some extent between the different syndecans.

Syntenin also binds to B-class ephrins (16, 17). In blotting experiments, the bindings of the various GST-syntenin fusion proteins to GST-ephrin closely mimicked their bindings to GST-syntenin-2 (Fig. 7). Since syndecan-2 interacts with the
PDZ domain of CASK (Fig. 6) and CASK also binds to neurexins. When the complete cytoplasmic domain of human neurexin-2 was used as bait in the two-hybrid system an interaction (both growth on His− and β-Gal staining) was observed. When GST-neurexin was immobilized on the blot and probed with GST-myc-syntenin fusion proteins in the overlay assay, we obtained clear binding signals not only with syntenin or the PDZ1 + PDZ2 tandem but also with the PDZ1 + PDZ1 tandem (Fig. 7). GST-syntenin fusion proteins containing a combination of the lysine and aB1 mutations in either PDZ1 or PDZ2 also bound to neurexin (Fig. 7). GST fusion proteins containing the combined lysine and aB1 mutations in both PDZ1 and PDZ2 did not bind to immobilized neurexin (Fig. 7). Thus, as observed for the interaction with syndecan, the interaction of syntenin with neurexins and B-class ephrins appears to depend on the cooperation of PDZ1 and PDZ2 but at least PDZ1 appears to have higher intrinsic affinity for neurexins than for syndecans and B-class ephrins.

To assess the spectrum of the major potential types of syntenin bait in cells, we also performed ligand overlay assays on blots of whole cell extracts obtained from heparitinase-treated human cells (Fig. 8). Only GST-syntenin fusion proteins that contained both PDZ1 and PDZ2 produced signals, binding to a series of prominent bands. Parallel staining of these blots with syndecan-specific antibodies (directed against epitopes in the cytoplasmic domains of these molecules) indicated that these bands corresponded to syndecan core proteins or core protein fragments. Thus, at least in these cells, syndecans figure among the quantitatively most important bait for the paired PDZ domains of syntenin.

**DISCUSSION**

The localization and clustering of cell surface receptors to specific subcellular positions can be critical for their proper functioning in the sending and receiving of signals. Syntenin is an adapter-like PDZ protein that binds to the cytoplasmic domain of a number of important signaling and adhesion molecules: syndecans (2), ephrins (16, 17), eph-receptors (16), and, as we show here, also neurexins. Deletion of either one of the two PDZ domains from syntenin abolishes or strongly reduces the interaction of syntenin with all the above partner proteins. Together with the present results, this suggests that the interaction of syntenin with all these proteins is PDZ-mediated but that its PDZ domains do not function as independent binding domains. Each domain needs the assistance of the other PDZ domain in a two-pronged binding that engages two contiguous PDZ domains and two contiguous bait peptides. In general, couples of PDZ domains whose interactions are weak taken individually would appear to be designed to bind to specific oligomeric structures rather than to drive the assembly of such structures. Syntenin could therefore be a “detector” of receptor clustering or co-clustering. Two compatible receptors that occur in synteny (the state of being together in location) together bind syntenin. The multiple in vitro interactions or potential partners of syntenin suggest that the PDZ domains of syntenin have a somewhat relaxed specificity. Yet, the promiscuity of syntenin appears limited, and the PDZ domains of syntenin cannot invariably be substituted one for the other in the various binding interactions with syndecans, ephrins, and neurexins. This lack of cross-complementation suggests that the two PDZ domains of syntenin differ somewhat in their affinities for a specific partner and possibly in their partner preferences. Syntenin may therefore bind to and discriminate between a number of specific supramolecular complexes or structures that include assemblies and mixed assemblies of syndecans and other suitable partners. Many ligands for cell surfaces with signal functions contain multiple binding domains, including heparin binding domains that could be instrumental in driving these syndecan assemblies.

PDZ domains are widespread among signaling and cytoskeletal proteins and are thought to localize the activities of these proteins to appropriate sub-membranous protein complexes
Most known PDZ domain interactions occur through recognition of short C-terminal peptide motifs, which include the terminal carboxylate of the ligand. Binding specificity is achieved by domain interactions with the residue at the -2 position of the bound peptide, serine/threonine (coordinating with histidines near the start of the αβ-helix) in the case of type-I domains and hydrophobic or aromatic residues (coordinating with non-basic residues in the αβ-helix) in the case of type-II domains. Syntenin binds to C-terminal peptide structures such as t-FYA (syndecans), t-YKV (ephrin-Bs), t-IQV (Eph A7), and t-YYV (neurexin) and features Ser as the αβ1 residue in PDZ1 and Asp as the αβ1 residue in PDZ2. Moreover, syntenin does not bind to t-SYA, as in syndecan-2.

FIG. 5. Isothermal titration calorimetry. The top panel shows the raw heat data corrected for base-line drift, obtained from 18 consecutive injections of 0.03 μmol of syndecan-2 peptide into a cell containing 0.13 μmol of syntenin (4.21 mg). The bottom panel shows the binding isotherm created by plotting the areas under the peaks against the molar ratio of syndecan-2 peptide added to syntenin present in the cell (boxes) and the fit (line) to a model involving two binding sites in syntenin.

FIG. 6. Syntenin and the PDZ domain of CASK bind differentially to the cytoplasmic domains of the syndecans. Ligand overlays with GST-myc-syntenin (full-length (FL)) and GST-myc-CASK PDZ domain fusion proteins. The blotted GST-syntenin fusion proteins represent the cytoplasmic domains of the four different syndecans (1–4). Bound ligand was detected with the anti-myc monoclonal antibody 9E10.
F(C303)S, indicating that at least one of its PDZ domains is a type-II domain. Mutations of either the carboxylate binding lysines or the α1 residues in either PDZ1 or PDZ2 all affect the syndecan-syntenin binding, and combinations of these mutations have additive negative effects on this binding. All this together is compatible with a bivalent binding model, engaging a syndecan dimer (acting as a plug) and the two type-II PDZ domains of syntenin (functioning as a socket).

Nonetheless, there is an increasing number of PDZ-mediated interactions that do not conform to the canonical mode of PDZ recognition and where the interaction is based on the recognition of internal peptide motifs. Crystal structures indicate that, in this case, a β-hairpin finger docks in the peptide binding groove, and a sharp β-turn replaces the normally required C terminus. Such alternative internal interaction modes support the heteromeric dimerization of the PDZ domain of neuronal nitric-oxide synthase with PDZ domains from PSD95 and syntrophin (15, 19). The demonstration of interactions between PDZ domains in heterodimeric complexes raises the further possibility that intramolecular PDZ-PDZ interactions may serve to regulate the conformations and functions of proteins with two or more PDZ domains (20). All this indicates that tandem arrays of PDZ domains may mediate multiple interactions and complicate the interpretation of the effects of deletions in terms of binding assignments. The effects of the point mutations that we introduced in the PDZ domains of syntenin confirm that directly or indirectly both domains are involved in the binding of syntenin to di- or multicmeric syndecans. A single PDZ domain is clearly insufficient for syndecan binding, but the effects of the mutations could also result from disruptions or weakening of homotypic PDZ-PDZ interactions that support the formation of syntenin dimers or multimers. In that case, it remains unclear what PDZ domain directly mediates binding to the immobilized syndecan peptide. PDZ1-supported syntenin dimers may bind to syndecans via PDZ2 (dimers), or alternatively, PDZ2-supported syntenin dimers may bind to syndecans via PDZ1 (dimers). The first of the two alternatives would be consistent with the binding modes observed for the various PDZ tandem constructs, since the PDZ1 + PDZ1 tandem fails to bind syndecans, whereas the PDZ2 + PDZ2 tandem does interact (although the latter could only be deduced from yeast two-hybrid experiments). However, full-length syntenin does not interact with full-length syndecan in yeast two-hybrid and surface plasmon resonance experiments (not shown). A full-length syntenin that is produced in a self-splicing intein expression system (which allows the production of recombinant protein that is free of any extensions such as GAL4 or GST) does not show any evidence for self-association in gel filtration and cross-linking experiments (not shown). Moreover, highly sensitive isothermal micro-calorimetry experiments, using syntenin produced from intein fusion proteins and free peptide, can detect a syntenin-syndecan interaction in solution and suggest a 1:2 syntenin-syndecan binding stoichiometry. All together, this strongly favors a model wherein stable binding results from the engagement of two intramolecular PDZ domains and two oligomerized syndecans. Although both PDZ domains of syntenin appear to directly participate in the binding to syndecan, the consistently more pronounced effects of the PDZ2 mutations suggest that PDZ2 contributes to a larger extent than PDZ1. Relative affinity estimates based on the current data can only be very tentative, but all together the results are compatible with a model where the contribution of PDZ2 is twice as strong as that of PDZ1, and binding to syndecan requires a combination of strengths that exceeds that of a single PDZ2 domain.

In the yeast two-hybrid assay, several bait fusion proteins are bound to the GAL4-responsive promoter and are, thus, automatically presented as a homotypic assembly. This display system would appear neutral with respect to the detection of PDZ domains that function in isolation and well suited for the detection of coupled PDZ domains with similar or shared specificities. Yet, it may be strongly negatively biased with respect to co-operative PDZ domains with distinct target specificities. Syndecan bait is very efficient at recruiting syntenin prey, which invariably contain the combination of PDZ1 + PDZ2 and typically represent 80% of the clones isolated from a hippocampal library screen. Unless over-represented in this library because of the relative abundance of the corresponding transcript, this result suggests that homotypic assemblies of syndecans selectively or preferentially recruit syntenin. The overlay experiments suggest that homotypic assemblies of ephrins or neurexins may do this with similar or even greater efficiencies. This does not ensure that this bait represents the optimal fit (and therefore potentially natural partners) for the syntenin-PDZ domains. At minimum, our failures to detect binding partners in yeast two-hybrid screens using single syntenin PDZ domains as bait2 and in ligand overlays of blotted cell extracts that use GST with a single syntenin PDZ1 domain as a probe support the contention that the PDZ domains of syntenin do not work in isolation. Yet, whereas strong binding to any of the arrayed bait requires the combination of PDZ1 and PDZ2, the relative contributions of PDZ1 and PDZ2 appear to not always mimic the hierarchy observed in the syndecan-syntenin binding. The separate mutations of PDZ1 and PDZ2 affect the binding to neurexin less differentially than they affect the binding to syndecan or ephrin-B1, and the combination of PDZ1 + PDZ1 binds to neurexin but not to syndecan or ephrin-B1. By inference this implies that at least PDZ1 adapts better to neurexins than to syndecans or B-class ephrins. Both PDZ domains of syntenin may have a somewhat relaxed specificity but differential preferences for different subsets of type-II C termini.

Differential peptide preferences of PDZ1 and PDZ2 imply that in a physiological setting syntenin may bind to specific heterotypic assemblies rather than to homotypic assemblies that would represent a less than optimal fit. As PDZ1 prefers neurexin over syndecan-2, the combination of a neurexin and a syndecan could potentially figure as an example of a supramolecular complex that represents a better fit for syntenin than homotypic syndecan assemblies. Neurexin is present at the presynaptic side of the neuronal synapse. Syndecan-2 accumulates in central neuronal synapses, where it appears to be specifically associated with both the postsynaptic density and the presynaptic terminal (21), indicating that from the known syndecan and neurexin expressions, this suggestion could make sense. Resolving this issue will require the identification of the bait that is physiologically associated with syntenin in cells. It is clear that there are also different PDZ proteins competing for the same bait. Both CASK and syntenin bind to neurexins and syndecans. Syntenin is widely expressed. CASK is appropriately expressed and localized to interact with both syndecan-2 and -3 in different compartments of the neuron throughout postnatal development (21). As we show here, the single PDZ domain of CASK binds to all four syndecans but clearly more easily to the syndecans -2 and -4 than to the syndecans -1 and -3, which respects the structural similarities among the syndecans. Indeed, the sequence immediately upstream of the EFYA terminus of syndecan-1 is highly similar to the corresponding region in syndecan-3, and the corresponding

2 J. J. Grootjans, G. Reekmans, H. Ceulemans, and G. David, unpublished results.
region in syndecan-2 is highly similar to that in syndecan-4 but more distantly related to these sequences in the syndecans -1 and -3. Syntenin, in contrast, does not show a similar preference and binds equally well to all four syndecans. Ligand overlays suggest that syntenin binds better to assemblies of syndecan or neurexin bait than CASK binds to this same bait, but we do not have data that allow a precise quantitative comparison of the relative strengths of the neurexin/syndecan-CASK and neurexin/syndecan-syntenin bindings. Yet, should these be of the right order, it is conceivable that regulated co-expressions and associations of syndecans and neurexins could result in a switch in transmembrane connections and signaling from CASK-mediated to syntenin-mediated modes. Such a recruitment model could fit in a broader “dual” receptor concept, where switches in cytoplasmic connections are supported by ligands with cooperative receptor binding domains that realize the required syntenies of the receptor cytoplasmic domains. Clearly, further investigations are needed to substantiate these proposals.

Acknowledgments—We are very grateful to Dr. Jan Backmann (Free University of Brussels) for his assistance in the isothermal titration calorimetry experiments and thank Dr. Pascale Zimmermann and Dr. Joachim Durr for their critical comments and assistance in the redaction of this manuscript.

REFERENCES
1. Bernfield, M., Gottle, M., Woo Park, P., Reizes, O., Fitzgerald, M. L., Lincecum, J., and Zako, M. (1999) Annu. Rev. Biochem. 68, 729–777
2. Grootjans, J. J., Zimmermann, P., Reekmans, G., Smets, A., Degeest, G., Durr, J., and David, G. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13683–13688
3. Cohen, A. R., Woods, D. F., Marfatia, S. M., Walther, Z., Chishti, A. H., Anderson, J. M., and Wood, D. F. (1999) J. Cell Biol. 142, 129–138
4. Hsueh, Y. P., Yang, F. C., Kharazia, V., Naisbitt, S., Cohen, A. R., Weinberg, R. J., and Sheng, M. (1998) J. Cell Biol. 142, 139–151
5. Fanning, A. S., and Anderson, J. M. (1999) J. Clin. Invest. 103, 767–772
6. Cahal, J. H., Petosa, C., Sutcliffe, M. J., Razo, S., Byron, O., Puy, F., Marfatia, S. M., Chishti, A. H., and Liddington, R. C. (1996) Nature 382, 649–652
7. Doyle, D. A., Lee, A., Lewis, J., Kim, E., Sheng, M., and MacKinnon, R. (1996) Cell 83, 1067–1076
8. Tochio, H., Hung, P., Li, M., Bredt, D. S., and Zhang, M. (2000) J. Mol. Biol. 295, 225–237
9. Daniels, D. L., Cohen, A. R., Anderson, J. M., and Brunger, A. T. (1998) Nat. Struct. Biol. 5, 317–325
10. Songyang, Z., Fanning, A. S., Fu, C., Xu, J., Marfatia, S. M., Chishti, A. H., Crompton, A., Chan, A. C., Anderson, J. M., and Cantley, L. C. (1997) Science 275, 73–77
11. Dong, H., O’Brien, R. J., Fung, E. T., Lamahan, A. A., Worley, P. F., and Huganir, R. L. (1997) Nature 386, 279–284
12. Lue, R. A., Brandin, E., Chan, E. P., and Branton, D. (1996) J. Cell Biol. 133, 1125–1137
13. Hata, Y., Butz, S., and Sudhof, T. C. (1996) J Neurosci 16, 2488–2494
14. Brennan, J. E., Chao, D. S., Gee, S. H., McGee, A. W., Craven, S. E., Santillano, D. R., Wu, Z., Huang, F., Xia, H., Peters, M. F., Frohner, S. C., and Bredt, D. S. (1996) Cell 84, 757–767
15. Hillier, B. J., Christopherson, K. S., Prehoda, K. E., Bredt, D. S., and Lim, W. A. (1995) Science 264, 812–815
16. Torres, R., Firestein, B. L., Dong, H., Staudeinger, J., Olsen, E. N., Huganir, R. L., Bredt, D. S., Gale, N. W., and Yancopoulos, G. D. (1998) Neuron 21, 1453–1463
17. Lin, D., Gish, G. D., Songyang, Z., and Pawson, T. (1999) J. Biol. Chem. 274, 3726–3733
18. Fanning, A. S., and Anderson, J. M. (1999) Curr. Opin. Cell Biol. 11, 432–439
19. Tochio, H., Zhang, Q., Mandai, P., Li, M., and Zhang, M. (1999) Nat. Struct. Biol. 6, 417–421
20. Gomperts, S. N. (1996) Cell 84, 659–662
21. Hsueh, Y. P., and Sheng, M. (1999) J. Neurosci. 19, 7415–7425
