The protein tyrosine phosphatases PTPRZ and PTPRG bind to distinct members of the contactin family of neural recognition molecules

Samuel Bouyain, and Dara J. Watkins

Division of Molecular Biology and Biochemistry, School of Biological Sciences, University of Missouri-Kansas City, Kansas City, MO 64110

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The receptor protein tyrosine phosphatases gamma (PTPRG) and zeta (PTPRZ) are expressed primarily in the nervous system and mediate cell adhesion and signaling events during development. We report here the crystal structures of the carbonic anhydrase-like domains of PTPRZ and PTPRG and show that these domains interact directly with the second and third immunoglobulin repeats of the members of the contactin (CNTN) family of neural recognition molecules. Interestingly, these receptors exhibit distinct specificities: PTPRZ binds only to CNTN1, whereas PTPRG interacts with CNTN3, 4, 5, and 6. Furthermore, we present crystal structures of the four N-terminal immunoglobulin repeats of mouse CNTN4 both alone and in complex with the carbonic anhydrase-like domain of mouse PTPRG. In these structures, the N-terminal region of CNTN4 adopts a horseshoe-like conformation found also in CNTN2 and most likely in all CNTNs. This restrained conformation of the second and third immunoglobulin domains creates a binding site that is conserved among CNTN3, 4, 5, and 6. This site contacts a discrete region of PTPRG composed primarily of an extended β-hairpin loop found in both PTPRG and PTPRZ. Overall, these findings implicate PTPRG, PTPRZ and CNTNs as a group of receptors and ligands involved in the manifold recognition events that underlie the construction of neural networks.

cell adhesion | crystal structure | Ig superfamily | receptor protein tyrosine phosphatase

The development and maintenance of the nervous system relies on adhesive interactions between cell surface receptors and the coordinated reversible phosphorylation of downstream protein effectors. The receptor protein tyrosine phosphatases (RPTPs) participate in these two seemingly disparate processes as they combine extracellular domains (ECDs) that resemble those of cell adhesion molecules (CAMs) with one or two intracellular tyrosine phosphatase domains (1). In particular, the proteins PTPRG and PTPRZ make up the type V subgroup of RPTPs and are expressed predominantly in the developing and adult brains of vertebrates (2–4). However, their expression patterns differ because PTPRG is found almost exclusively on neurons, whereas PTPRZ localizes to glial cells. Each of these type I transmembrane proteins includes a domain homologous to α-carbonic anhydrases (CAs) (5), a single fibronectin type III repeat followed by a cysteine-free spacer, a membrane-spanning region, and tandem cytoplasmic tyrosine phosphatase domains (6, 7).

No ligand for PTPRG has been reported, but several binding partners for PTPRZ have been described and can be grouped according to the portion of the ECD that they recognize. The growth factor pleiotrophin binds to the spacer region as well as a glycosaminoglycan insert found in some isoforms of PTPRZ (8). The Ig superfamily CAMs L1/Ng-CAM, N-CAM, Nr-CAM associate with the spacer region, whereas the IgCAM protein contactin1 (CNTN1/contactin/F3) binds to the CA domain (9, 10). Importantly, the association between PTPRZ on glia and CNTN1 on neurons promotes the outgrowth of neurites and induces bidirectional signaling between glia and neurons suggesting that this interaction plays a role in nervous system development (9, 11). However, the structural basis for these interactions remains unclear.

CNTN1 is a glycosphatidylinositol (GPI)-anchored protein comprised of 6 N-terminal Ig domains followed by four fibronectin type III domains. It belongs to a family of neural recognition molecules involved in the patterning of neural tissues that includes five additional receptors sharing approximately 40–60% sequence identity (Table S1, 12, 13): CNTN2 (TAG-1/axonin), CNTN3 (PANG/BIG-1), CNTN4 (BIG-2), CNTN5 (NB-2), and CNTN6 (NB-3). As a first step toward illuminating the roles of type V RPTPs and CNTNs in establishing neuronal connections we undertook biochemical and structural studies of PTPRG, PTPRZ, and CNTNs.

Results

Crystal Structures of CA Domains from PTPRG and PTPRZ. The crystal structures of mouse PTPRGCA, human PTPRGCA and human PTPRZCA were determined by molecular replacement and refined to 1.7 Å (Rwork = 17.3%, Rfree = 20.5%), 1.7 Å (Rwork = 17.8%, Rfree = 19.3%), and 2.0 Å (Rwork = 17.5%, Rfree = 21.9%), respectively (Table S2). These CA-like domains are similar and contain a central 8-strand antiparallel β-sheet surrounded by three to four α-helices and extensive loop regions (Fig. 1 and Fig. SL4 and B). Mouse and human PTPRGCA superimpose with a rmsd of 0.5 Å for 257 Cα positions, whereas mouse PTPRGCA and human PTPRZCA superimpose with a rmsd of 1.4 Å for 257 Cα positions. However, unlike PTPRGCA, PTPRZCA includes an additional disulfide bond between C133 and C264, which is conserved in all known orthologs of PTPRZ. The presence of this additional disulfide bridge has little effect on the structure of PTPRZCA when compared to PTPRGCA and its biological significance is currently unknown.

Overall, the CA domains of PTPRG and PTPRZ resemble bona fide α-CAs. Mouse PTPRCAG, human PTPRGCA, and human PTPRZCA superimpose with mouse CA-XIV with rmsd of 1.5–1.7 Å for 253–255 Cα positions and most of the differences reside in the orientation of an extended β-hairpin loop in the type V RPTPs (Fig. 1D, 14). This hairpin loop is disordered in human PTPRGCA and in three of the four molecules found in the asymmetric unit of mouse PTPRGCA crystals, indicating that this region is flexible. In contrast the hairpin is ordered in both copies of human PTPRZCA found in the crystallographic asymmetric unit presumably because it participates in lattice interactions.

Author contributions: S.B. designed research; S.B. and D.J.W. performed research; S.B. analyzed data and wrote the paper.

The authors declare no conflict of interest.

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 3JXA, 3JXF, 3JXG, 3JXH, 3KLD).

*To whom correspondence should be addressed. E-mail: bouyains@umkc.edu.

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The catalytic site of α-CAs is characterized by the presence of three histidine residues that coordinate a zinc ion essential for enzymatic activity (5). This cavity is still present in PTPRG and PTPRZ but only one of the histidine residues is conserved (Fig. S1C); consequently no electron density indicative of a bound metal ion was observed.

PTPRG Interacts with CNTN Family Members. Because the CA domain of PTPRZ binds to CNTN1, we predicted that the CA domain of PTPRG could also mediate protein–protein interactions. Moreover, we predicted that these domains could bind similar molecules, such as members of the CNTN family. Therefore, an in vitro affinity-isolation assay was designed to probe the binding of type V RPTPs to CNTNs. Mouse GPI-anchored CNTNs were fused to hGH and expressed transiently in HEK293 cells. Cell lysates containing detergent-solubilized CNTNs were then incubated with mouse PTPRG and mouse PTPRGCA immobilized on sepharose. We confirmed the interaction between PTPRZCA and CNTN1 described previously (9), but found no indication of PTPRZCA-binding to other CNTNs (Fig. 2A). In line with previous studies, no interaction between PTPRGCA and CNTN1 was detected (9). However, PTPRGCA bound to CNTN3, 4, 5, and 6 (Fig. 2B). These interactions have not been described previously.

Because of the sequence similarities between type V RPTPs on one hand and CNTNs on the other hand and because type V RPTPs bind to CNTN family members, we hypothesized that PTPRGCA and PTPRGCA could interact with homologous subdomains of CNTNs. To test this hypothesis, we first identified the region of CNTN1 that mediates binding to PTPRZ. Secreted hGH-tagged fragments of mouse CNTN1 were incubated with a mouse PTPRGCA affinity resin. A complete CNTN1 ECD lacking the GPI anchor, a truncated form of CNTN1 including Ig domains 1–4, and a form of CNTN1 lacking Ig domains 1–4 were tested initially (Fig. 3A). The results show that the first four Ig domains bind to PTPRGCA. Further dissection showed that single Ig domains are unable to support binding to PTPRGCA, but that a fragment composed of Ig domains 2 and 3 of CNTN1 is necessary and sufficient to interact with immobilized PTPRGCA (Fig. 3B). The PTPRG-binding site on CNTN3, 4, 5, and 6 was localized using a similar approach. We confirmed that PTPRGCA bound to Ig domains 1–4 of CNTN3, 4, 5, and 6 but not those of CNTN1 or 2 (Fig. S2). Fragments of CNTN4 containing single and overlapping pairs of Ig domains within the first four Ig repeats were then analyzed (Fig. 3B). As was observed in the case of PTPRZ and CNTN1, Ig repeats 2 and 3 make up the minimal PTPRGCA-binding site on CNTN4. These domains are also sufficient to bind to PTPRGCA in the case of CNTN3, 5, and 6 (Fig. 3C). Taken together, these results indicate that CNTN proteins bind to distinct type V RPTPs, but that the binding site spans the second and third Ig repeats in all cases tested.

Because interactions detected by affinity-isolation assays may depend on additional factors found in cell lysates or conditioned media, we tested whether type V RPTPs associate directly with CNTNs. The interaction between PTPRGCA and CNTN1 was thus further studied by analytical size-exclusion chromatography (Fig. 3D). This experiment showed that the two molecules form a 1:1 complex at a concentration of 10 μM. It was not possible to purify significant amounts of CNTN1[1–4] to perform a similar experiment with PTPRGCA so we used CNTN4[1–4] instead. As was observed for PTPRZ and CNTN1, PTPRGCA and CNTN4[1–4] form a 1:1 complex (Fig. 3E). No evidence of higher oligomer formation was observed in either case.

Ig Domains 1–4 of Mouse CNTN4 Adopt a Horseshoe-Like Conformation. CNTN2, the only member of the CNTN family that does not bind to the CA domains of either PTPRZ or PTPRG, adopts a horseshoe-like conformation (15, 16). To determine if significant structural differences between CNTN2[1–4] and CA-binding CNTNs could account for this observation, we determined the crystal structure of mouse CNTN4[1–4] to 2.4 Å resolution.

![Fig. 1. Structures of CA domains from type V RPTPs.](Image)

A Human PTPRZ

B Human PTPRG

C Mouse PTPRG

D Mouse PTPRGCA

![Fig. 2. Affinity isolation of GPI-anchored CNTNs.](Image)

A CNTN1

B CNTN2

C CNTN3

D CNTN4

E CNTN5

F CNTN6

G Bound

H Input

I Beads: PTPRZ

J Beads: PTPRG
Residues F171, Y180, and A182 in Ig2 and V229 and F248 in Ig3 (Fig. 4). Taken together, these observations suggest that all CNTN family members may adopt a similarly conformation. T aken together, these observations suggest that all CNTN family members may adopt a similarly conformation. The interface between Ig pairs 1-2 and 3-4 is mostly apolar and involves essentially two discrete segments, residues 129–142 in Ig2 and 220–228 in Ig3, which partially overlap with the regions that

\( R_{\text{work}} = 19.2\% \), \( R_{\text{free}} = 25.2\% \), Table S2). As was observed for CNTN2, contacts between Ig domains 1 and 4 on one hand and 2 and 3 on the other hand lock CNTN4 into a horseshoe-like conformation (Fig. 4A and Fig. S3). The structures of human and chicken CNTN4 and mouse CNTN4 in fact resemble each other fairly closely despite limited sequence identity (rmsd of 1.6–2.3 Å, Table S1). Similar conformations have been reported for the insect immune protein hemolin and isoforms of the Down syndrome cell adhesion molecule (DSCAM) (17–19).

The interface between Ig pairs 1-2 and 3-4 is mostly apolar and buries a surface area of 2,233 Å\(^2\) with a shape complementarity coefficient of 0.72, both of which compare favorably to those of known biological interfaces (20, 21). Its core includes residues F171, Y180, and A182 in Ig2 and V229 and F248 in Ig3 (Fig. 4B). Residues F171, Y180, and F248 are absolutely conserved in human and mouse CNTNs (Fig. S4); A182 is changed to S in CNTN2, which still adopts an identical conformation.

The β-hairpin Loop of PTPRGCA Mediates Binding to Ig Domains 2 and 3 of CNTN4. We determined the crystal structure of a complex of PTPRGCA and CNTN4 into 2.0 Å resolution (\( R_{\text{work}} = 17.9\% \), \( R_{\text{free}} = 23.2\% \), Table S2) to provide a structural basis for the interactions between type V RPTPs and CNTNs (Fig. 5). The asymmetric unit contains a single 1:1 complex of PTPRGCA and CNTN4 and analysis of the lattice contacts did not indicate the presence of higher order oligomers, which is consistent with our size exclusion chromatography experiments (Fig. 3E). In PTPRGCA, the flexible β-hairpin (residues 288–301) accounts for approximately 80% of the interactions with CNTN4; the remainder of the contacts are mediated by residues 225–229. In line with our affinity-isolation assays (Fig. 3B), contacts between CNTN4 and PTPRGCA are limited to Ig domains 2 and 3. The structures of Ig domains 2 and 3 in both the free and bound states superimpose with a rmsd of 0.8 Å for 191 C\(^\alpha\) atoms, indicating that only minimal structural changes occur upon binding of PTPRG. Interestingly, the PTPRG-binding site in CNTN4 involves essentially two discrete segments, residues 129–142 in Ig2 and 220–228 in Ig3, which partially overlap with the regions that

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The β-hairpin Loop of PTPRGCA Mediates Binding to Ig Domains 2 and 3 of CNTN4. We determined the crystal structure of a complex of PTPRGCA and CNTN4 into 2.0 Å resolution (\( R_{\text{work}} = 17.9\% \), \( R_{\text{free}} = 23.2\% \), Table S2) to provide a structural basis for the interactions between type V RPTPs and CNTNs (Fig. 5). The asymmetric unit contains a single 1:1 complex of PTPRGCA and CNTN4 and analysis of the lattice contacts did not indicate the presence of higher order oligomers, which is consistent with our size exclusion chromatography experiments (Fig. 3E). In PTPRGCA, the flexible β-hairpin (residues 288–301) accounts for approximately 80% of the interactions with CNTN4; the remainder of the contacts are mediated by residues 225–229. In line with our affinity-isolation assays (Fig. 3B), contacts between CNTN4 and PTPRGCA are limited to Ig domains 2 and 3. The structures of Ig domains 2 and 3 in both the free and bound states superimpose with a rmsd of 0.8 Å for 191 C\(^\alpha\) atoms, indicating that only minimal structural changes occur upon binding of PTPRG. Interestingly, the PTPRG-binding site in CNTN4 involves essentially two discrete segments, residues 129–142 in Ig2 and 220–228 in Ig3, which partially overlap with the regions that
mediate homophilic binding in DSCAMs (Fig. S5). This suggests that the horseshoe-like scaffold can support homophilic and heterophilic binding modes using homologous surfaces.

The interface between PTPRGCA and CNTN4[α1-4] buries a total of 1,702 Å² with a shape complementarity coefficient of 0.67, values similar to those of antibody–antigen complexes (1, 680 Å² and 0.64–0.68, respectively, 20, 21). The two strands of the β-hairpin in PTPRGCA complement the 3-strand antiparallel β-sheet in CNTN4[βi] to form a 5-strand antiparallel β-sheet with the main chain atoms of residues 295–299 of PTPRG forming hydrogen bonds with the main chain atoms of residues 139–143 in CNTN4 (Fig. 5). The interface contains a hydrophobic region consisting of V132, L142, M220, Y223, and L250 in CNTN4 and F288, V296, and V299 in PTPRG as well as a hydrophilic region consisting of V132, L142, M220, Y223, and L250 in CNTN4 and K297, and E300 in PTPRG (Fig. 6). In addition to the contacts mediated by the β-hairpin, the side chain of H226 in PTPRG abuts the aliphatic portion of K226 in CNTN4 and K229 mediates potential salt bridges and hydrogen bonds with CNTN4 residues E224 and N304 (Fig. 6). All the CNTN4 residues that interact with PTPRG are conserved in CNTN3, 5, and 6, thus explaining why PTPRG binds specifically to these four CNTN family members (Fig. S4).

The structure of the PTPRG · CNTN4 complex likely approximates the complex between PTPRZ and CNTN1. Indeed, because Ig2 and Ig3 in CNTN1 probably adopt a restrained conformation similar to the one of CNTN4 and because these domains are necessary and sufficient to mediate binding to PTPRZCA, the PTPRZ-binding site on CNTN1 likely spans a region similar to the PTPRG-binding site on CNTN4. Amino acid changes at key positions help rationalize the absence of detectable binding between PTPRZ and CNTN3, 4, 5, and 6. Residues G273 and M276 in PTPRZ replace residues D294 and K297 in PTPRG resulting in the loss of polar interactions with CNTN4 residues S130 and E228 (Fig. 6 and Fig. S1). Sequence variations within the CNTN family also explain, in part, why PTPRG does not interact with CNTN1 or 2. M220 is replaced by T in CNTN1 and by P in CNTN2; the hydrogen bond/salt bridge between CNTN4 residues E228 and K297 in PTPRG is not possible in CNTN1 (E is changed to V) or CNTN2 (E is changed to K). CNTN4 residue Q138 is replaced by W in CNTN2 and therefore unable to form a hydrogen bond with E300 of PTPRG. In addition, CNTN4 residue S130 is changed to D in CNTN2, possibly resulting in charge repulsion with D294 in PTPRG (Fig. 6).

Discussion

The interactions Between PTPRG and CNTN3, 4, 5, and 6 Identified In Vitro May Occur In Vivo. The biochemical and structural data presented here suggest that PTPRG and PTPRZ are potential ligands for all but one member of the CNTN family of IgCAMs. The binding between PTPRZ and CNTN1 has already been described (9), but the interactions between PTPRG and CNTN3, 4, 5, and 6 have not. Although indirect, several lines of evidence suggest that these interactions are relevant in vivo.

PTPRG is expressed in the nervous system of mouse embryos and in the adult brain (4). Expression of PTPRG is restricted to neurons and is found in adult mouse pyramidal cells; it is also detected in all sensory organs, including the glomeruli of the olfactory bulb. Recently it was demonstrated that CNTN4 is expressed in some but not all olfactory sensory neurons in mouse and is important for their migrating axons to target the glomeruli of the olfactory bulb (22). Furthermore, these same studies detected but did not identify a heterophilic binding partner for CNTN4 expressed on the glomeruli. The data presented here in

Fig. 5. Structure of the PTPRGCA · CNTN4[α1-4] complex. The view on the left is obtained from the one shown in Fig. 4A by a counterclockwise rotation of approximately 60° along a vertical axis. In the right view, only residues 225–229 and 288–301 (β-hairpin) of PTPRGCA are shown for the sake of clarity. The letters N and C indicate the N- and C-termini, respectively. Disulfide bonds are shown as orange ball-and-stick models. Asparagine-linked N-acetylgalcosamine residues are depicted as gray ball-and-stick models along with the asparagine side chain. Ig domains 1, 2, 3, and 4 are colored cyan, green, gold, and red, respectively. PTPRGCA is colored magenta. Dotted lines indicate disordered regions. The β-hairpin region is well ordered with average B factors of 32.1 Å² versus 33.8 Å² for the entire chain of PTPRGCA.

Fig. 6. Stereo view of the PTPRGCA · CNTN4[α1-4] interface. This view is in the same orientation as the right view in Fig. 5. Residues are shown as ball-and-sticks with transparent gray spheres for those involved in van der Waals contacts. Dashed lines indicate potential hydrogen bonds and salt bridges. Residues from CNTN4[β2], CNTN4[β1], and PTPRGCA are colored green, gold, and magenta, respectively.
addition to the expression pattern of PTPRG suggest that PTPRG could be the CNTN4-binding protein found in the olfactory bulb. Although less is known about the function of CNTN3, its expression in the olfactory bulb of adult mice matches the expression pattern of PTPRG indicating that a physical interaction is possible in vivo (4, 23).

Both PTPRG and CNTN6 are expressed in layer V of the cerebral cortex and in the pyramidal field CA1 of the hippocampus. Interestingly, mice deficient in either PTPRG or CNTN6 exhibit impaired motor coordination during rod walking and string tests (4, 24). These similar phenotypes suggest that the physical association of PTPRG and CNTN6 may be important for acquiring proper motor functions. CNTN5 is expressed early postbirth and its expression is the strongest in regions involved in the auditory pathway, in particular in the cochlear nuclei (25; 26). This localization of CNTN5 matches that of PTPRG (4), which is also found, at least at the embryonic stage, in the nuclei of the vestibulocochlear nerve responsible for carrying signals about hearing and balance.

An Indirect Control of Diphosphorylation? One of the most important questions about RPTPs concerns the functional relationship between the ECD and the control of the phosphatase activity. Biochemical and structural lines of evidence suggest that dimerization of RPTPs is linked to inhibition of the phosphatase activity. In particular, PTPRZ is a catalytically active monomer on the cell surface in the absence of the growth factor pleiotrophin but becomes inactive after treatment with pleiotrophin induces dimerization of the ECD (27). Furthermore, the tandem phosphatase domains of PTPRG form stable dimers in solution, which occludes the catalytic site (28). Taken together these observations provide strong evidence that dimerization of type V RPTPs can control their catalytic activity.

Interestingly, our experiments show that the formation of the PTPRZCA · CNTN1Ig2–3 and PTPRGCA · CNTN4Ig2–3 complexes does not induce dimerization of either PTPRZ or PTPRG (Fig. 3D, E and Fig. 5). One reason could be that larger fragments of PTPRG, PTPRZ, or CNTNs are required to bring about changes in oligomeric state upon extracellular binding. However, an alternative explanation is that binding by CNTNs does not control phosphatase activity directly but rather specifies the location of dephosphorylation reactions. Indeed, PTPRM (RPTPr), a type IIa RPTP, forms head-to-tail dimers that localize specifically to regions of cell–cell contacts where the intercellular spacing matches the length of the PTPRM dimer (29). A possible role for CNTNs could then be to guide PTPRZ and PTPRG toward specific cellular regions, thus providing a spatial control of dephosphorylation (30).

Materials and Methods
Detailed descriptions are given in SI Materials and Methods.

Protein Expression and Purification. Human and mouse PTPRGCA (residues 56–320) and PTPRGCA (residues 34–302) were expressed a fusion proteins with thioredoxin, a hexahistidine tag, and a human rhinovirus 3C protease site in E. coli (residues 34–250) or Origami B(DE3). Fusion proteins were purified by immobilized-metal affinity chromatography. After cleavage with human rhinovirus 3C protease, proteins were purified by ion exchange and size exclusion chromatography.

GPI-anchored and secreted fragments of CNTN proteins were transiently expressed in HEK293 cells as fusion proteins with hGH, a octahistidine tag, and a human rhinovirus 3C protease site. Mouse CNTN1Ig2–3 (residues 133–239) and CNTN4Ig2–3 (residues 25–404) used for analytical size exclusion chromatography experiments were purified from the conditioned media of transiently transfected HEK293 cells by immobilized-metal affinity chromatography followed by proteolytic removal of the hGH fusion partner and ion exchange chromatography. Deglycosylated CNTN4Ig2–3 was prepared for structural analysis by substituting N-acetylgalactosaminyltransferase I-negative HEK293S cells for HEK293 cells. N-linked carbohydrates were removed using endoglycosidase H.

Affinity-Isolation Assays. Purified mouse PTPRGCA and PTPRZCA were coupled to CNBr-activated sepharose at a density of 1 mg of protein per ml of resin, respectively. For each reaction 250 μl of conditioned medium containing a secreted mouse CNTN fragment was mixed with 250 μl of 50 mM Tris-HCl pH 7.4, 200 mM NaCl, and 0.2% (vol/vol) Tween-20 and incubated for 1 h at room temperature with 30 μl of a 50% (vol/vol) slurry of PTPRZCA or PTPRGCA resin for 2 h at room temperature. Resins were washed with 25 mM Tris-HCl pH 7.4, 100 mM NaCl and 0.1% (vol/vol) Tween-20. Samples were resolved by SDS-PAGE and bound CNTN fragments were detected by immunoblotting using a rabbit polyclonal antibody against hGH ( Fitzgerald).

For affinity-isolation assays using membrane-bound CNTNs, transfected cells were detached from the plastic plate in PBS containing 5 mM EDTA, harvested by centrifugation and suspended in 100 mM NaCl, 10% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, 1% (vol/vol) sodium deoxycholate, 50 mM Tris-HCl pH 8.0, protease inhibitors. After lysis on ice, cellular debris were removed by centrifugation and lysates were cleared with 30 μl of a 50% (vol/vol) slurry of quenched CNBr-activated sepharose for 1 h at room temperature. Lysates were then incubated with 20 μl of a 50% (vol/vol) slurry of PTPRGCA or PTPRZCA for 2 h at room temperature. Resins were washed with 25 mM Tris-HCl pH 7.4 and 100 mM NaCl. Samples were resolved by SDS-PAGE and mouse CNTNs were detected by immunoblotting against hGH.

Characterization of Binding Interactions Using Size Exclusion Chromatography. A mixture of mouse PTPRZCA (3 nmol) and mouse CNTN1Ig2–3 (3 nmol) was adjusted to 300 μl with PBS and incubated for 2 h at 4 °C. An aliquot (200 μl) of this mixture was analyzed by size exclusion chromatography using a Superdex 200 10/30 HR column (GE Healthcare) in PBS. The same protocol was used to probe the binding between PTPRGCA and CNTN4Ig2–3.

Crystalization and Structure Determination. All crystals were grown at 20 °C by hanging drop vapor diffusion. Oscillation diffraction data were collected at 1.00 A for beams 23-ID or 22-8M of the Advanced Photon Science of Argonne National Laboratory and processed with HKL2000 (31).

The structure of mouse PTPRGCA was solved by molecular replacement with PHASER as implemented in PHENIX using the structures of human CA-XII and mouse CA-XIV in a single ensemble as a search model (14, 32, 33). The final model was obtained after manual building using COOT and refinement in PHENIX (32, 34). It consists of four chains with residues 58–181, 185–293, and 295–320 in molecule A, residues 58–292 and 297–320 in molecule B, residues 57–320 in molecule C, residues 58–291 and 298–320 in molecule D, and 689 water molecules.

The structures of human PTPRGCA and human PTPRZCA were solved by molecular replacement using the structure of mouse PTPRGCA as a search model. The final model for human PTPRGCA consists of residues 58–291, 298–320, 145 water molecules, and one sulfate ion. The final model for human PTPRZCA includes residues 34–301 in molecules A and B as well as 222 water molecules.

The structure of CNTN4Ig2–3 was determined by molecular replacement using chicken CNTN2Ig3 and CNTN2Ig3 as search models (35). The final model consists of two protein chains with residues 25–40, 43–404 in molecule A, residues 25–404 in molecule B, 8 N-acetylgalactosamine residues, and 189 water molecules. Structural descriptions in the text refer to molecule A because it has overall lower B factors than molecule B.

The structure of a complex of mouse PTPRGCA and mouse CNTN4Ig2–3 was determined by molecular replacement with PHENIX using the structures of mouse PTPRGCA, mouse CNTN4Ig2–3, and mouse CNTN4Ig2–3 as independent search models (35). The final model consists of residues 25–71, 76–402, and 4 N-acetylgalactosamine residues in CNTN4Ig2–3, residues 56–95 and 99–320 in PTPRGCA, and 415 water molecules.

Ramachandran statistics were calculated using RAMPAGE as implemented in CCP4 (35). Core residues from protein structures were superimposed using the Dalili server (36). Buried surface areas and lists of contact residues were calculated in CCP4; surface complementarity coefficients were obtained using the program SC (20). Figures were prepared with PyMOL (www.pymol.org).
Cloning, expression, and purification of mouse contactins (CNTNs). DNA fragments encoding mouse CNTN1 (residues 21–1020), CNTN2 (residues 36–1040), CNTN4 (residues 25–1026), and CNTN6 (residues 26–1028) were amplified from clones purchased from Open Biosystems and cloned in the same modified pET32 plasmid. Expression plasmids were transformed in Escherichia coli strain Origami 2(DE3) (Novagen) except the plasmid encoding mouse PTPRZCA, which was transformed in Escherichia coli strain Origami B(DE3) (Novagen). To express each protein, an aliquot of an overnight culture of transformed bacteria (20 mL) was used to inoculate 1 L of Luria-Bertani broth containing 100 μg/mL of ampicillin (and 50 μg/mL of kanamycin in the case of Origami B(DE3) cells), and the culture was grown at 30 °C and 225 rpm. Expression of targeted proteins was induced when OD_600 reached 0.8 by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.5 mM. The temperature was decreased to 25 °C and cells were grown for a further 16–18 h. Harvested cells were suspended in 50 mM of loading buffer (500 mM NaCl, 10 mM imidazole, and 40 mM sodium phosphate, pH 7.8) and lysed by microfluidization. The lysate was centrifuged 30 min at 30,000 x g, and the supernatant was applied to a 4-mL column of chelating sepharose (GE Healthcare) previously charged with nickel sulfate. The column was washed with loading buffer and bound proteins were eluted with loading buffer containing 250 mM imidazole. After cleavage with human rhinovirus 3C protease and dialysis against loading buffer, the proteins were passed over a His-Trap column (GE Healthcare). The flow through was dialyzed against 20 mM Tris-HCl, pH 8.0, 20 mM NaCl and the proteins were purified by affinity chromatography, the N-linked carbohydrates were removed by incubating the protein with endoglycosidase H for 3 h at 30 °C followed by size-exclusion chromatography using a Superdex 200/26/60 column equilibrated in 20 mM Tris-HCl, pH 8.0 and 200 mM NaCl. Fractions containing deglycosylated CNTN4e9e–11 were pooled, dialyzed against 40 mM sodium phosphate, pH 7.0, and purified by ion exchange on a Resource S column as described above. The yield of purified, deglycosylated CNTN4e9e–11 was approximately 2 mg/L of conditioned media.

Secreted fragments of mouse CNTNs fused to hGH used in affinity-isolation assays were produced in 6-well plates by transient transfections of HEK293S cells for the transfection (2). These cells only produce high-mannose N-linked glycans. After purification of CNTN4e9e–11 by immobilized metal affinity chromatography, the N-linked carbohydrates were removed by incubating the protein with endoglycosidase H for 3 h at 30 °C followed by size-exclusion chromatography using a Superdex 200/26/60 column equilibrated in 20 mM Tris-HCl, pH 8.0 and 200 mM NaCl. Fractions containing deglycosylated CNTN4e9e–11 were pooled, dialyzed against 40 mM sodium phosphate, pH 7.0, and purified by ion exchange on a Resource S column as described above. The yield of purified, deglycosylated CNTN4e9e–11 was approximately 2 mg/L of conditioned media.

Crystallization and structure determination. All crystals were grown at 20 °C by hanging drop vapor diffusion.

Mouse PTPRCα. Protein solution (1 μL of a 7 mg/mL solution in 2 mM Tris-HCl pH 8.0 and 20 mM NaCl) was mixed with 1 μL of a 3:1 mixture of water: reservoir buffer [100 mM Na-HEPES pH 7.5, 20% (wt/vol) polyethylene glycol (PEG) 10,000] Prior to data collection, crystals were transferred to a solution of reservoir buffer supplemented with 15% (vol/vol) PEG 400 and flash cooled in liquid nitrogen.

Diffraction data were collected at 1.00 Å at beamline 22-ID of the Advanced Photon Source of Argonne National Laboratory and processed with HKL2000 (3). The structure was solved by molecular replacement with Phaser as implemented in PHENIX using the structures of mouse CA-XII and mouse CA-XIV in a single ensemble as a search model (4–6). An initial model was obtained with the auto-build wizard from PHENIX. The fusion of hGH and CNTN4e9e–11 was expressed and purified essentially the same way, but using a 6-mL Resource Q column equilibrated in 20 mM Tris-HCl pH 8.0, 20 mM NaCl instead of the Resource S column. Deglycosylated CNTN4e9e–11 was prepared by employing N-acetylglucosaminyltransferase I-negative HEK293S cells for the transfection (2). These cells only produce high-mannose N-linked glycans. After purification of CNTN4e9e–11 by immobilized metal affinity chromatography, the N-linked carbohydrates were removed by incubating the protein with endoglycosidase H for 3 h at 30 °C followed by size-exclusion chromatography using a Superdex 200/26/60 column equilibrated in 20 mM Tris-HCl, pH 8.0 and 200 mM NaCl. Fractions containing deglycosylated CNTN4e9e–11 were pooled, dialyzed against 40 mM sodium phosphate, pH 7.0, and purified by ion exchange on a Resource S column as described above. The yield of purified, deglycosylated CNTN4e9e–11 was approximately 2 mg/L of conditioned media.

Secreted fragments of mouse CNTNs fused to hGH used in affinity-isolation assays were produced in 6-well plates by transient transfections of HEK293S cells using 2 μg of plasmid mixed with 4 μg of polyethyleneimine for each well. Conditioned media were collected 4 days after transfection and clarified by centrifugation. The amount of hGH in each lot of conditioned media was assessed by immunoblotting against hGH followed by quantitation using a Typhoon fluorimager. Using this procedure, we were able to perform the affinity-isolation assays using similar amounts of hGH fusion proteins in each reaction.
The final model was obtained after several rounds of manual model building using COOT and refinement in PHENIX (4, 7). The final model consists of four chains with residues 58–181, 185–293, and 295–320 in molecule A, residues 297–297 in molecule B, residues 57–320 in molecule C, residues 58–291 and 298–320 in molecule D, and 689 water molecules.

**Human PTPRGCA.**
Protein solution (1 μL of a 6.5 mg/mL solution in 2 mM Tris–HCl pH 8.0 and 20 mM NaCl) was mixed with 1 μL of a 3:1 mixture of water:reservoir buffer [50 mM Na-citrate pH 6.0, 150 mM (NH₄)₂SO₄ and 10% (wt/vol) PEG 4,000]. Crystals were then transferred to a solution of reservoir buffer supplemented with 25% (vol/vol) PEG 400 and flash cooled in liquid nitrogen.

Diffraction data were collected at 1.00 Å at beamline 22-ID of the Advanced Photon Source of Argonne National Laboratory and processed with HKL2000 (3). The structure was solved by molecular replacement with PHASER as implemented in PHENIX using the structure of mouse PTPRGCA as a search model. The final model was obtained after manual model building using COOT and refinement in PHENIX and consists of residues 58–291 and 297–298, 145 water molecules, and 1 sulfate ion (4, 7).

**Human PTPRZCA.**
Protein solution (2 μL of a 2 mg/mL solution in 10 mM Tris–HCl pH 8.0 and 100 mM NaCl) was mixed with 1 μL of 100 mM Na-cacodylate pH 6.5, 200 mM Mg(OAc)₂ and 20% (wt/vol) PEG 8,000). Plate clusters appeared within 2 days. They were crushed and used as microseeds to obtain single plates. For data collection, crystals were transferred to a solution of reservoir buffer supplemented with 15% (vol/vol) glycerol and flash cooled in liquid nitrogen.

Diffraction data were collected at 1.00 Å at beamline 22-ID of the Advanced Photon Source of Argonne National Laboratory and processed with HKL2000 (3). The structure was solved by molecular replacement with Phaser as implemented in PHENIX using the structure of mouse PTPRGCA as a search model. The final model was obtained after manual model building using COOT and refinement in PHENIX and consists of residues 25–250, 145 water molecules, and 1 sulfate ion.

**Mouse CNTN₄⁴₉₄–⁵–⁴.**
Protein solution (1 μL of a 3.5 mg/mL solution in 2 mM Tris–HCl pH 8.0 and 100 mM NaCl) was mixed with 1 μL of a 20 mM Na/K-phosphate pH 5.8, 1.2 M 1,6-hexanediol and 10% (wt/vol) PEG 3,400. Crystals were transferred to a solution of 20 mM Na/K-phosphate pH 5.8, 1.2 M 1,6-hexanediol and 20% (wt/vol) PEG 3,400 and flash cooled in liquid nitrogen prior to data collection.

Diffraction data were collected at 1.00 Å on the same crystal at beamlines 22-BM and 22-ID of the Advanced Photon Source of Argonne National Laboratory, processed with HKL2000 and merged (3). The crystals belong to space group C2 with two molecules in the asymmetric unit. The structure was determined by molecular replacement with PHENIX using domains Ig1-2 and Ig3-4 of chicken CNTN2 as independent search models (8). Initially, only one copy of CNTN₄⁴₉₄–⁵–⁴ was found. This model was subjected to one round of rigid-body refinement in PHENIX using each Ig repeat as a single body. The resulting structure was then used as a search model and the two copies of CNTN₄⁴₉₄–⁵–⁴ were found in the asymmetric unit. The final model was obtained after manual model building using COOT and refinement in PHENIX (4, 7). The final model consists of residues 25–40, 45–404 in molecule A, residues 25–404 in molecule B, 8 N-acetylglucosamine residues (four in each chain), and 189 water molecules. The two CNTN₄⁴₉₄–⁵–⁴ molecules in the asymmetric unit superimpose with rmsd of 2.2 Å for 376 shared Cα atoms, but pairs of domains are more closely related: rmsd of 1.2 Å for Ig domains 1 and 4 and of 0.6 Å for domains 2 and 3.

**Mouse PTPRGCA · CNTN₄⁴₉₄–⁵–⁴.**
Purified mouse PTPRGCA and mouse CNTN₄⁴₉₄–⁵–⁴ were mixed in a 1:1 ratio and concentrated to 20 μM in 10 mM Tris–HCl pH 8.0 and 100 mM NaCl. An aliquot (1 μL) was mixed with 1 μL of 50 mM Na-cacodylate pH 7.0 and 10% (wt/vol) PEG 1,500. Crystals appeared within 5–7 days. Crystals were transferred to a solution of 50 mM Na-cacodylate pH 7.0, 10% (wt/vol) PEG 1,500, and 20% (vol/vol) PEG 400 and flash cooled in liquid nitrogen prior to data collection.

Diffraction data were collected at 1.00 Å at beamline 22-BM of the Advanced Photon Source of Argonne National Laboratory and processed with HKL2000 (3). The crystals belong to space group P2₁2₁2₁ with one heterodimer per asymmetric unit. The structure was determined by molecular replacement with PHENIX using the structures of mouse PTPRGCA, mouse CNTN₄⁴₉₄–⁵–⁴, and mouse CNTN₄⁴₉₄–⁵–⁴ as independent search models. The final model was obtained after manual model building using COOT and refinement in PHENIX (4, 7). The final model consists of residues 25–71, 76–402, and 4 N-acetylglucosamine residues in CNTN₄⁴₉₄–⁵–⁴, residues 56–95 and 99–320 in PTPRGCA, and 415 water molecules.
Fig. S1. Overview of the mouse PTPRGCA structure. (A) Stereo view of the mouse PTPRGCA structure. The letters N and C indicate the N and C termini, respectively. Disulfide bonds are shown as orange ball-and-stick models. (B) Same as (A), but this view is related to the view in (A) by a 90° rotation clockwise along a vertical axis. (C) Sequence alignment of CA domains from human and mouse PTPRG, PTPRZ, CA-XII, and CA-XIV. The numbering refers to mouse PTPRG. Strictly conserved residues are shaded in black and similar residues are colored gray. The two cysteine residues involved in the single disulfide bond in PTPRG are numbered in green below the sequences. Black triangles indicate the two cysteine residues that form the additional disulfide bridge found in PTPRZ. Open circles indicate the positions of the histidine residues that coordinate a zinc ion necessary for catalytic activity in CA-XII and CA-XIV. Green and gold circles below the sequences indicate the residues involved in key interactions with domains Ig2 and Ig3 in CNTN4, respectively. The two strands of the β-hairpin that mediate most of the interactions with CNTN4 in PTPRG are shown in magenta.
Fig. S2. Binding of PTPRG to the first four Ig domains of CNTN family members. The first four Ig domains of mouse CNTNs were fused to hGH and expressed transiently in HEK293 cells. Conditioned media were incubated with PTPRG resin and bound proteins were visualized by immunoblotting against hGH.

Fig. S3. Stereo view of the horseshoe-like conformation of mouse CNTN4Ig1-4. The letters N and C indicate the N and C termini, respectively. Disulfide bonds are shown as orange ball-and-stick models. Asparagine-linked N-acetylglucosamine residues are depicted as gray ball-and-stick models along with the asparagine side chain. Ig domains 1, 2, 3, and 4 are colored cyan, green, gold, and red, respectively.
Fig. S4. Alignment of amino acid sequences of Ig domains 1–4 of human and mouse CNTNs. Strictly conserved residues are shaded in black and similar residues are colored gray. The numbering refers to mouse CNTN4. Cysteine residues involved in disulfide bridges are numbered in green below the sequences. Blue triangles indicate the residues involved in key interactions between Ig domains 2 and 3 in the horseshoe-like structure of CNTN4. Magenta circles indicate residues involved in PTPRG binding in CNTN4 as observed in the PTPRG · CNTN4 complex.
Fig. S5. Homologous binding regions in the horseshoe-like structures of CNTN4 and DSCAM. (A) Ribbon diagrams and surface representations of the horseshoe-like structures. Residues involved in heterophilic binding (CNTN4, Left) or homophilic binding (DSCAM, Right) are shown in red. The positions of these residues are shown in red in the structure-based sequence alignment below. (B) Sequence alignment of the first four Ig domains of mouse CNTN4 and DSCAM D1–D49. The numbering refers to the mouse CNTN4. Strictly conserved residues are shaded in black and similar residues are colored gray. The red lines above and below the sequences indicate the amino acid segments involved in binding interactions in CNTN4 and DSCAM, respectively. These amino acid positions correspond to the residues highlighted in red in (A).
Table S1. Amino acid sequence identities in mouse CNTNs

<table>
<thead>
<tr>
<th></th>
<th>CNTN1</th>
<th>CNTN2</th>
<th>CNTN3</th>
<th>CNTN4</th>
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<th>CNTN6</th>
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<td>70</td>
<td>58</td>
<td></td>
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</table>

The amino acid sequences of mouse CNTNs were aligned using CLUSTALW and the percentage of amino acid identity for the full-length proteins (top) and the second and third Ig domains (bottom, bold) were calculated.

Table S2. Data collection and refinement statistics

<table>
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<th>Beamline</th>
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<th>APS 22-ID</th>
<th>APS 22-ID</th>
<th>APS 22-ID, 22-BM</th>
<th>APS 22-BM</th>
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<td>Crystal</td>
<td>Mouse PTPRGCA</td>
<td>Human PTPRGCA</td>
<td>Human PTPRZCA</td>
<td>Mouse CNTN</td>
<td>Mouse PTPRGCA · CNTN</td>
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<tr>
<td>Wavelength, Å</td>
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<td>1.00</td>
<td>1.00</td>
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<tr>
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<td>46,797</td>
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<tr>
<td>Resolution, Å</td>
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<td>50–1.7</td>
<td>50–2.0</td>
<td>50–2.4</td>
<td>40–2.0</td>
</tr>
<tr>
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<td>P2₁</td>
<td>P2₁2₁</td>
<td>P2₁</td>
<td>C2</td>
<td>P2₁2₁</td>
</tr>
<tr>
<td>Unit cell a, b, c, Å</td>
<td>82.76, 85.14, 91.07</td>
<td>52.54, 66.17, 86.76</td>
<td>63.96, 68.12, 65.68</td>
<td>281.73, 40.43, 95.08</td>
<td>100.43, 138.96, 50.57</td>
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<tr>
<td>α, β, γ, °</td>
<td>115.71, 90.00</td>
<td>90.00</td>
<td>99.9 (99.3)</td>
<td>90.00</td>
<td>95.8 (80.3)</td>
</tr>
<tr>
<td>R_sym*</td>
<td>0.088 (0.510)</td>
<td>0.093 (0.484)</td>
<td>0.115 (0.381)</td>
<td>0.136 (0.548)</td>
<td>0.156 (0.475)</td>
</tr>
<tr>
<td>Completeness, %</td>
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<td>97.8 (82.1)</td>
<td>99.9 (99.3)</td>
<td>97.0 (81.1)</td>
<td>95.8 (80.3)</td>
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<tr>
<td>Redundancy</td>
<td>3.6 (2.8)</td>
<td>12.0 (5.6)</td>
<td>3.6 (2.9)</td>
<td>9.2 (4.1)</td>
<td>12.8 (8.5)</td>
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<tr>
<td>I/σ (I)</td>
<td>12.7 (1.9)</td>
<td>22.1 (2.7)</td>
<td>10.4 (3.1)</td>
<td>13.1 (2.0)</td>
<td>14.6 (3.6)</td>
</tr>
</tbody>
</table>

Refinement

| Number of molecules in asymmetric unit | 4 | 1 | 2 | 2 | 1 |
| Number of protein atoms | 8,347 | 2,078 | 4,311 | 5,984 | 5,067 |
| Number of water atoms | 689 | 145 | 222 | 189 | 415 |
| rms deviation from ideal bonds, Å | 0.009 | 0.007 | 0.006 | 0.005 | 0.011 |
| rms deviation from ideal angles, ° | 1.206 | 1.076 | 0.969 | 0.895 | 1.354 |
| Average B factors, Å² | 32.9 | 39.1 | 34.8 | 78.6 | 36.5 |
| Main chain | 28.3 | 34.3 | 29.8 | 76.6 | 32.0 |
| Side chain | 36.3 | 42.9 | 39.4 | 81.6 | 40.1 |
| Water | 40.0 | 44.1 | 39.1 | 51.8 | 37.2 |

Ramachandran statistics

| Favorable, % | 98.7 | 97.6 | 97.9 | 94.2 | 96.2 |
| Allowed, % | 1.3 | 2.4 | 2.1 | 5.8 | 3.8 |

‡R = Σ|obs(h) − F(calc)(h)|/ΣF(obs)(h), Rwork and Rfree were calculated from the working and test reflection sets, respectively. The test set constituted 5% of the total reflections not used in refinement.

* R_sym = Σ|I(h)|/Σmax |I(h)|, where I(h) is the |i|th measurement of reflection h and <I(h)> is a weighted mean of all measurements of h.
†Values in parentheses apply to the high-resolution shell.