PTPσ functions as a presynaptic receptor for the glypican-4/LRRTM4 complex and is essential for excitatory synaptic transmission

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Leukocyte common antigen-related receptor protein tyrosine phosphatases—comprising LAR, PTPδ, and PTPσ—are synaptic adhesion molecules that organize synapse development. Here, we identify glypican 4 (GPC-4) as a ligand for PTPσ. GPC-4 showed strong (nanomolar) affinity and heparan sulfate (HS)-dependent interaction with the Ig domains of PTPσ. PTPσ bound only to proteolytically cleaved GPC-4 and formed additional complex with leucine-rich repeat transmembrane protein 4 (LRRTM4) in rat brains. Moreover, single knockdown (KD) of PTPσ, but not LAR, in cultured neurons significantly reduced the synaptogenic activity of LRRTM4, a postsynaptic ligand of GPC-4, in heterologous synapse-formation assays. Finally, PTPσ KD dramatically decreased both the frequency and amplitude of excitatory synaptic transmission. This effect was reversed by wild-type PTPσ, but not by a HS-binding–defective PTPσ mutant. Our results collectively suggest that presynaptic PTPσ, together with GPC-4, acts in a HS-dependent manner to maintain excitatory synapse development and function.

PTPσ | glypican | LRRTM4 | synaptic cell adhesion | heparan sulfate

Synaptic adhesion molecules orchestrate every aspect of synapse development, and certain synaptic adhesion proteins, called "synapse organizers," coordinate the structure and function of mammalian synapses (1, 2). The leukocyte common antigen-related receptor protein tyrosine phosphatases (LAR-RPTPs), which comprise three members in vertebrates (LAR, PTPδ, and PTPσ), have recently emerged as synapse organizers (3). They mediate presynaptic differentiation via various postsynaptic ligands (3). All three members of the LAR-RPTP family bind to netrin-G ligand 3 (4): PTPδ binds to interleukin 1-receptor accessory protein-like 1 (5); PTPσ binds to TrkC (6); and PTPδ and PTPσ, but not LAR, bind to the Slit- and Trk-like proteins (7, 8). However, we do not yet understand the molecular basis for these various binding modes.

The above-mentioned LAR-RPTP ligands exist only in vertebrates, arguing that their synaptic adhesion pathways do not account for all of the evolutionarily conserved synaptic functions of the LAR-RPTPs. Indeed, studies have shown that invertebrate LAR-RPTP orthologs (dLAR in *Drosophila melanogaster* and PTP-3 in *Caenorhabditis elegans*) are crucial for nervous system development and function in such organisms (9). The candidates for the evolutionarily conserved LAR-RPTP ligands include the glypican (GPC) family of heparan sulfate proteoglycans (HSPGs), which are linked to the cell membrane via a glycosylphosphatidylinositol (GPI) anchor (10). The GPCs comprise six members (GPC-1 to -6) in mammals, two [Dally and Dally-like protein (Dlp)] in fruit fly, and one (GPN-1) in worm (11). In fruit fly, Dlp and another HSPG syndecan (Sdc) function redundantly to regulate midline axon guidance (12), but perform distinct functions in synapse development via binding to dLAR (13, 14). In vertebrates, GPCs are predominantly expressed during nervous system development, where they serve as guidance cues for axonal navigation and neuronal migration. Consistent with the role in axon guidance, GPCs also interact with the axon guidance molecule Slit (15, 16). Moreover, they regulate several signaling pathways, including the Wnt, Hedgehog, fibroblast growth factor, and bone morphogenic protein pathways (17). Furthermore, GPCs are proteolytically cleaved by a furin-like convertase, generating two subunits that are attached by one or more disulfide bonds, although the requirement for this processing for various GPC functions has not been clearly established (18). We do not yet fully understand the mechanisms through which GPCs function at mammalian synapses, but they were recently found to be presynaptic receptors for leucine-rich repeat transmembrane protein 4 [LRRTM4 (refs. 19 and 20; see also ref. 21)]. Given that GPCs

Significance

This paper documents and systematically characterizes the molecular interactions of protein tyrosine phosphatase σ (PTPσ) with glypicans (GPCs). The identified interactions require heparan sulfate (HS), suggesting that GPCs are a major source of HS for PTPσ at excitatory synapses. Strikingly, we found that leucine-rich repeat transmembrane protein 4 (LRRTM4) induces presynaptic differentiation via the PTPσ/GPC interaction, suggesting that PTPσ may function as a coreceptor for GPCs in presynaptic neurons. More importantly, we found that HS-binding ability of PTPσ is critical for excitatory synaptic transmission. These results expand our previous understanding of how synaptic adhesion pathways regulate excitatory synapse development and shed light on GPCs/LRRTM4 *trans*-synaptic signaling. Moreover, to our knowledge, this is the first study to document the physiological significance of HS in the presynaptic function of mammalian neurons.

Author contributions: J.K. designed research; J.S.K., G.P., J.W.U., J.S.S., D.L., K.H.K., and G.C. performed research; G.-Y.C. and J.d.W. contributed new reagents/analytic tools; J.S.K., G.P., J.W.U., J.S.S., K.H.K., H.M.K., H.K., J.d.W., K.-S.P., K.T., and J.K. analyzed data; and J.S.K., J.W.U., and J.K. wrote the paper.

The authors declare no conflict of interest.

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are GPI-anchored, it seems likely that yet-unidentified presynaptic membrane protein(s) may act as coreceptors for the GPC/LRRTM4 interaction.

Here, we show that GPC-4 binds PTPσ with high affinity in a heparan sulfate (HS)-dependent manner. Intriguingly, PTPσ binds only to cleaved GPC-4, which is highly expressed at an early stage of brain development, and further associates with LRRTM4 in postnatal and adult brains. More importantly, the HS-binding property of PTPσ is functionally critical for the synaptogenic action of LRRTM4 and for excitatory synaptic transmission in cultured neurons. Thus, we herein show, to our knowledge, for the first time that PTPσ is a bona fide presynaptic receptor for the GPC-4/LRRTM4 synaptic adhesion complex.

Results

GPC-4 Is a Potential Ligand for PTPσ. To identify additional ligand(s) for LAR-RPTPs in vivo, we chose to search for ligands of PTPσ because it is the most highly expressed LAR-RPTP in rat brain (22). We generated an expression vector that encoded the PTPσ extracellular domains fused to the Fc domain of human Ig (PTPσ–IgC) and one encoding the human Fc domain alone as a control protein (IgC; Fig. 1*A*). We immobilized these Ig fusion proteins and performed affinity chromatography using rat brain synaptosomes (Fig. 1*B*). The proteins from each silver-stained gel slice that showed a distinct band were purified on immobilized PTPσ and subjected to mass spectrometry (MS) (Fig. 1*B*). Among the identified peptides (Table S1), three were derived from GPC-4 (Fig. 1 *C* and *D* and Fig. S1 *A* and *B*). Previously, GPC-2 was shown to bind to PTPσ to promote outgrowth of dorsal root ganglion neurons (23); however, whether other GPCs also bind PTPσ has not been determined. First, to validate whether GPC-4 directly binds to PTPσ on the cell surface, we performed binding assays between recombinant Ig-fusion proteins of GPC-4 (Ig–GPC-4) and HEK293T cells expressing HA-tagged PTPσ (Fig. 1*E*). EGFP-fused LRRTM4 (Fig. 1*E*; ref. 19) and mVenus-fused LRRTM2 were expressed in HEK293T cells as positive and negative controls, respectively (Fig. 1*E*). We found that PTPσ and LRRTM4 avidly bound to GPC-4, whereas LRRTM2 did not (Fig. 1*E*). No interaction was observed between LRRTM4 and neurexin-1β recombinant proteins (Fig. S1*C*), which is consistent with our previous observation that excess soluble neurexin-1β recombinant proteins do not inhibit the synaptogenic activity of LRRTM4 (19). We did, however, observe an interaction between neurexin-1β and LRRTM4 recombinant proteins (Fig. S1*D*), suggesting that these proteins interact with each other under certain conditions (19). In addition, GPC-4 failed to show binding to any other cell-surface protein examined, indicating that it forms a specific interaction with PTPσ (Fig. S2). To estimate the binding affinity between GPC-4 and PTPσ, we incubated HA-PTPσ–expressing and control HEK293T cells with increasing amounts of Ig–GPC-4 and measured the cell-surface–bound proteins with an HRP-tagged secondary antibody. After we subtracted the nonspecific binding, we performed Scatchard analysis, assuming a single independent binding site for GPC-4 in each PTPσ molecule, and obtained a K_d of 41.3 ± 3.6 nM (Fig. 1*F*). Although this finding should be interpreted with some caution because the used dimeric GPC-4 ligands can produce an increased interaction affinity, our results indicate that GPC-4 binds to PTPσ with high affinity.

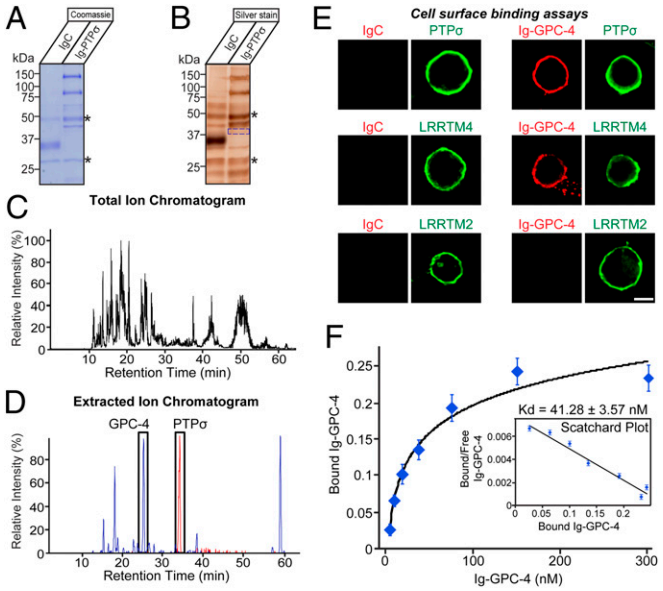

Fig. 1. Affinity purification of GPC-4 as a PTPσ-ligand in rat brain. (*A*) Coomassie blue-stained gel of recombinant Ig-control and Ig–PTPσ fusion proteins used for affinity chromatography. (*B*) Solubilized rat synaptosomes were subjected to pull-down assays with IgC or Ig–PTPσ. A blue box indicates a specific band unique to the Ig–PTPσ-bound fraction. Asterisks indicate the cleaved Ig–PTPσ proteins or IgC heavy chain. (*C* and *D*) Total ion chromatogram of a liquid chromatography (LC) separation of Ig–PTPσ-bound eluates (*C*). (*D*) Extracted ion chromatograms of ion *m/z* 972.03 and 517.28 from PTPσ (34.05 min) and GPC-4 (24.88 min). (*E*) Cell-surface binding assays. HEK293T cells expressing HA-PTPσ, LRRTM4–EGFP, or LRRTM2–mVenus were incubated with control IgC or Ig–GPC-4 and analyzed by immunofluorescence imaging for Ig-fusion proteins (red) and HA/mVenus (green). [Scale bar: 15 μm (applies to all images).] (*F*) Saturation binding of Ig–GPC-4 to PTPσ expressed in HEK293T cells. *Inset* shows a Scatchard plot generated by linear regression of the data, with the K_d calculated from three independent experiments. Data are presented as means ± SEM.

PTPσ Ig Domains Are Required for the Interaction with GPC-4. To determine which PTPσ domains interact with GPC-4, we performed cell-surface binding assays using constructs expressing full-length PTPσ (PTPσ-full), its Ig domains alone (PTPσ–Ig), or an Ig-domain-deleted protein (PTPσ–ΔIg) (Fig. 2*A*). IgC–GPC-4 bound to HEK293T cells expressing PTPσ-full and -Ig, but not those expressing PTPσ–ΔIg (Fig. 2*B*). Recent studies have established that alternative splicing events at the Ig domains of LAR-RPTPs determine their binding affinity toward postsynaptic ligands (3, 5, 6). Thus, we examined whether the GPC-4/PTPσ interaction is regulated by similar alternative splicing inserts (Fig. 2*C*). GPC-4 strongly bound to all four splice variants of PTPσ and three splice variants of LAR (Fig. 2 *C* and *D*), but showed weaker binding to all four splice variants of PTPδ (Fig. 2*E*). To assess the affinities of these GPC-4 interactions with PTPσ variants, we expressed PTPσ variants on the surfaces of HEK293T cells and estimated the binding affinity of different PTPσ isoforms (Fig. 2*F* and Fig. S3*A*). All PTPσ variants displayed nanomolar affinities comparable to that of the PTPσ-full (Fig. 2*F* and Fig. S3*A*). Moreover, all LAR and PTPδ variants bound to GPC-4 recombinant proteins with similar nanomolar affinities (Fig. S3 *B* and *C*). These data suggest that alternative splicing of the LAR-RPTPs does not regulate their binding affinity for GPC-4 per se.

The GPC-4/PTPσ Interaction Occurs in the Same Cell Membrane. Our observation of a GPC-4/PTPσ interaction in cell-surface binding assays (Figs. 1 and 2) raised the possibility that such binding could mediate *trans*-cellular adhesion, as observed in synapses. Therefore, we performed cell-adhesion assays (Fig. 3 *A* and *B*). We prepared L cells (red fluorescent cells) expressing DsRed alone (control) or coexpressing DsRed with PTPσ, and L cells (green fluorescent cells) expressing EGFP alone (control) or coexpressing EGFP with GPC-4 or TrkC. These cells were mixed and incubated for up to 60 min, and cell aggregation was measured (Fig. 3*B*). Quantification revealed that GPC-4–expressing cells did not form any aggregated clumps with PTPσ-expressing cells (Fig. 3 *A* and *B*). In contrast, TrkC-expressing cells formed

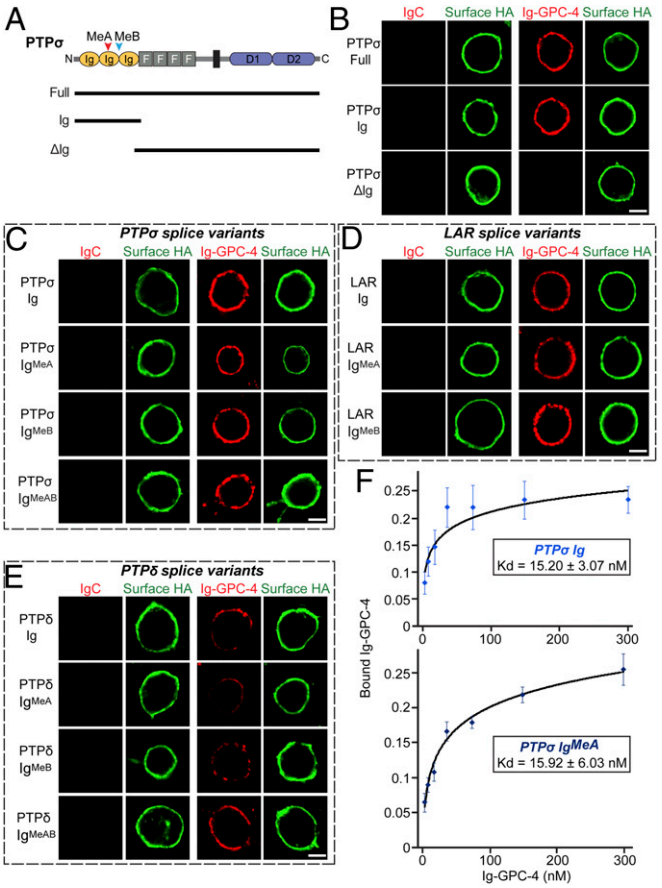

Fig. 2. Analysis of the GPC-4–binding domain of LAR-RPTPs. (*A*) Diagrams of the PTPσ vectors used in the cell-surface binding assays. F, fibronectin type III (FNIII) domains; D1, phosphatase domain 1 (catalytically active); D2, phosphatase domain 2 (catalytically inactive). (*B*) HEK293T cells expressing HA-tagged PTPσ-full (a splice variant of PTPσ that lacks in insert in splice sites MeA and MeB), PTPσ–Ig, or PTPσ–ΔIg were incubated with IgC or IgC–GPC-4 and analyzed by immunofluorescence imaging for the Ig-fusion proteins (red) and surface-exposed HA-PTPσ proteins (green). (*C–E*) HEK293T cells expressing the indicated splice variants of LAR-RPTPs Ig1–3 were incubated with IgC or Ig–GPC-4 and analyzed by immunofluorescence for the Ig-fusion proteins (red) and surface-exposed HA-tagged LAR-RPTPs (green). [Scale bars, *B–E*: 10 μm (applies to all images).] (*F*) Saturation binding of Ig–GPC-4 to a subset of PTPσ splice variants (PTPσ Ig and PTPσ Ig^{MeA}) expressed in HEK293T cells. See also Fig. S3 for the other splice variants of PTPσ. Data are presented as means ± SEM.

strong aggregations with PTPσ-expressing cells, which is consistent with their reported *trans*-interaction (ref. 6; Fig. 3 *A* and *B*). These data suggest that the GPC-4/PTPσ interaction mainly occurs in the *cis*-configuration.

PTPσ Requires HS to Interact with GPC-4. PTPσ was previously shown to bind the HS chains of HSPGs, such as agrin and collagen XVIII, and to colocalize with HSPGs on sensory neurons (23, 24). Therefore, we next examined whether the HS chains of GPC-4 mediate their binding to PTPσ. We first generated a PTPσ construct (PTPσ–AAAA) in which four lysines of the first Ig domain (K68, K69, K71, and K72) were all replaced with alanines to abrogate HS binding (Fig. 3*C* and ref. 23). We then expressed either PTPσ wild-type (WT) or PTPσ–AAAA in HEK293T cells, treated the cells with heparinase III (hep III; 1 U/mL) for 2 h to remove the HS chains from PTPσ, and stained the cells with a monoclonal 3G10 antibody that reacts only with hep III-treated HS chains (25). We found that cells expressing PTPσ WT, but not PTPσ–AAAA, reacted to the 3G10 antibody (Fig. 3*D*). To further examine whether the HS chains attached to PTPσ are required for the interaction with GPC-4, we treated PTPσ WT-expressing HEK293T cells with hep III and then used hep III-treated Ig–GPC-4 in cell-surface binding assays (Fig. 3*E*). Hep III treatment drastically reduced the binding of hep III-treated Ig–GPC-4 to PTPσ (Fig. 3*E*), suggesting the HS chains of PTPσ are required for this interaction. Consistently, PTPσ–AAAA failed to bind to GPC-4 (Fig. 3*E*). Whether the Ig1 domain of PTPσ was necessary and sufficient for GPC-4 binding could not be demonstrated because of poor surface transport of the PTPσ Ig1 construct (Fig. S4 *A* and *B*). Together, these data unequivocally demonstrate that the HS-binding ability of PTPσ is required for its interaction with GPC-4 (Fig. 3*E*). Lastly, because many cell-adhesion interactions, including those mediated by neurexins/neuroligins or neurexins/LRRTM2, require extracellular Ca^{2+} ions (26, 27), we tested whether the GPC-4/PTPσ interaction requires Ca^{2+}. However, we found that the Ca^{2+} chelator EGTA did not influence their binding (Fig. 3*F*). This finding suggests that the interaction of PTPσ with GPC-4 is Ca^{2+}-independent, analogous to the interactions of PTPσ with other ligands (4, 6).

PTPσ Interacts with Cleaved GPCs. To further corroborate the GPC-4/PTPσ interaction (Figs. 1–3), we performed pull-down assays using Ig–GPC-4 or IgC (negative control) against lysates from HEK293T cells expressing HA-PTPσ, HA-PTPσ AAAA, HA-LAR, HA-PTPδ, or NL1–mVenus. Ig–GPC-4 captured all three LAR-RPTP isoforms, but not PTPσ–AAAA or NL1 (Fig. 3*G*). We also used Ig–PTPσ or IgC to conduct pull-down assays with HEK293T cells expressing HA-tagged GPCs. The GPC-1 and -5 vectors were not efficiently processed in the HEK293T cells, because no bands were observed around ~37 kDa in HA–GPC-1– or HA–GPC-5–transfected cell lysates; in contrast, the other GPCs showed both ~65-kDa bands representing uncleaved species and ~37-kDa bands representing cleaved species (Fig. 3*H*). Our pull-down assays showed that the immobilized Ig–PTPσ effectively bound to cleaved GPCs, but not uncleaved GPCs (Fig. 3*H*). We also confirmed this finding using Ig–GPC-4 351-AISA (in which the furin-like convertase cleavage consensus $R^{351}ISR^{354}$ of GPC-4 was changed to $A^{351}ISA^{354}$), showing that the uncleaved recombinant GPC-4 proteins do not interact with PTPσ (Fig. S4*C*; see also Fig. S5). This binding property differs from that of the GPC-4/LRRTM4 interaction, where both uncleaved and cleaved GPCs bind to LRRTM4 (Fig. S4*D*) (19). Surprisingly, SDC-2 and -3, members of another HSPG family, failed to interact with PTPσ (Fig. S5*A*), suggesting that LAR-RPTPs use an evolutionarily distinct strategy for mediating synaptic adhesion; whereas dLAR binds both GPCs and SDCs, mammalian LAR-RPTPs prefer GPCs (28). Consistent with our above-described results (Fig. 3*E*), a GPC-4 mutant [GPC-4 AAA, in which the HS-attachment sites of GPC-4 (S494/S495/S500) were all mutated; ref. 19] showed no interaction with PTPσ in pull-down assays (Fig. 3*H*). We also observed a significant degree of enrichment of GPC-4, LRRTM4, and TrkC—but not NL1 or GluA1 (additional negative controls)—in the PTPσ-bound fraction of detergent-solubilized postnatal day 7 (P7) and P42 rat brain membrane fractions in pull-down assays using Ig–PTPσ fusion proteins (Fig. S5*B*). In addition, LRRTM4 antibodies coprecipitated with GPC-4 and PTPσ in coimmunoprecipitation assays performed on P7 rat brain membrane fractions (Fig. S5*C*). Notably, proteolytic cleavage of GPC-4 was developmentally regulated, exhibiting decreased cleavage during postnatal development (Fig. S5*D*), and only cleaved GPC-4 formed complexes and cofractionated with PTPσ in rat brains (Fig. S5 *B* and *E*), in accordance with the results from pull-down assays in HEK293T cells (see also Fig. 3*H*). Moreover, we showed that Ig–PTPσ bound to LRRTM4 expressed in HEK293T cells in the presence of GPC-4 WT, but not in the presence of GPC-4 AAA

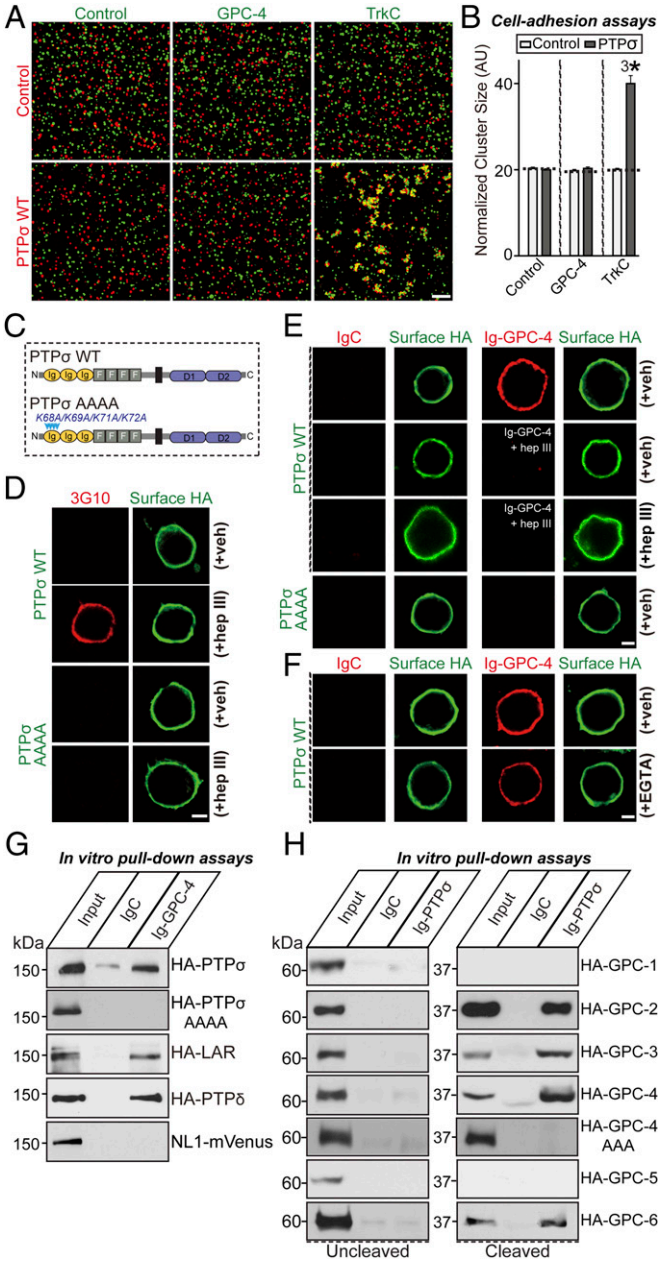

Fig. 3. PTPσ interacts with GPC-4 in a HS-dependent manner. (*A* and *B*) Representative images (*A*) and quantification of data (*B*) from cell-adhesion assays. HEK293T cells expressing EGFP alone (control) or coexpressing PTPσ with EGFP were mixed with HEK293T cells expressing DsRed alone (control) or coexpressing GPC-4 or TrkC with DsRed. The cells were imaged, and aggregations were quantified. [Scale bar: 100 μm (applies to all images).] $^{3}*P < 0.001$. (*C*) Schematic diagrams of PTPσ constructs used in *D* and *E*. (*D*) HEK293T cells were transfected with the indicated PTPσ vectors and treated with vehicle (+veh) or 1 U/mL heparinase III (+hep III). The cells were then stained with HA antibody (green) to detect surface-exposed PTPσ (red) under nonpermeabilized conditions. They were then permeabilized and stained with the 3G10 antibody. (*E*) Transfected HEK293T cells were treated with vehicle or hep III, incubated with IgC or IgC–GPC-4, and then analyzed by double-immunofluorescence microscopy for Ig-fusion proteins (red) and surface-exposed HA-PTPσ (green). (*F*) As in *E*, except that cells were also incubated with 10 mM EGTA (+EGTA). [Scale bars, *D*–*F*: 10 μm (applies to all images).] (*G* and *H*) Pull-down assays were performed with IgC and Ig–GPC-4 (*G*) or Ig–PTPσ (*H*) by using HEK293T cells expressing the indicated vectors (input: 1% of total). The numbers on the left indicate molecular mass markers (kDa). Immunoblot analyses showed that GPCs are expressed in two positions: a ~65-kDa band representing the full-length HA–GPCs (uncleaved) and a ~37-kDa band representing the N-terminal proteolytic fragment (cleaved).

(Fig. S5*F*). These results suggest that PTPσ specifically forms complexes containing GPCs and LRRTM4 (inferred through GPCs) in vivo.

PTPσ Is Required for LRRTM4-Mediated Presynaptic Differentiation in Cultured Neurons. Based on the direct interaction of GPCs with LAR-RPTPs (this work), the direct interaction of GPCs with LRRTM4 (19, 20) and the biochemical nature of GPCs as GPI-anchored proteins, we hypothesized that LAR-RPTPs might be functional presynaptic receptors for LRRTM4. To directly address this hypothesis, we used previously characterized lentiviral short-hairpin interference RNAs (shRNAs) against PTPσ (sh-PTPσ) or LAR (sh-LAR) [ref. 8; see also Fig. S5*G* for validation of PTPσ knockdown (KD) at the protein level]. Cultured hippocampal neurons were infected with control lentivirus (control) or lentiviruses expressing sh-LAR or -PTPσ, or coinfected with lentiviruses expressing LAR-KD and PTPσ-KD (sh-LAR/sh-PTPσ), and various heterologous synapse-formation assays were performed with infected neurons and HEK293T cells expressing LRRTM2, LRRTM4, or EGFP alone (control) (Fig. 4). PTPσ-KD alone, but not LAR-KD alone, significantly reduced the synaptogenic activity of LRRTM4, but not that of LRRTM2 (Fig. 4). These data are consistent with a previous report that LRRTM2 requires neurexins for its synaptogenic activity (29). Reexpression of PTPσ WT (+PTPσ WT) completely reversed the deficit in the synapse-formation activity of LRRTM4 observed in LAR/PTPσ-deficient neurons (Fig. 4), whereas reexpression of PTPσ–AAAA (+PTPσ–AAAA) did not. The data suggest that the HS-dependent interactions of PTPσ with GPCs are essential for inducing the presynaptic differentiation elicited by LRRTM4 (Figs. 3*E* and 4). We confirmed our previous observation that GPC-4 KD led to a significant deficit in the synaptogenic activity of LRRTM4 (19) (Fig. S6 *A* and *B*). This impairment of LRRTM4 activity was rescued by reexpression of GPC-4 WT, but not by expression of GPC-4 351-AISA (Fig. S6 *A* and *B*), consistent with our pull-down data showing that only cleaved GPC-4 bound to PTPσ (Fig. 3). These results collectively suggest that PTPσ acts via GPCs to function as a presynaptic receptor for LRRTM4.

The HS-Binding Sequence of PTPσ Is Essential for Excitatory Synaptic Transmission in Cultured Neurons. Most of the LAR-RPTP ligands have been demonstrated to cluster presynaptic vesicles and neurotransmitter release machineries by directly interacting with individual LAR-RPTP isoforms in the axons of cocultured neurons when expressed in heterologous cells, suggesting that LAR-RPTPs act as hubs for presynaptic organization (3). This notion, together with the failure of PTPσ–AAAA to restore the synaptogenic activity of LRRTM4 (Fig. 4), prompted us to ask whether the HS-binding property of LAR-RPTPs is also involved in some presynaptic functions (e.g., neurotransmitter release or synaptic transmission). Strikingly, no previous study had examined whether LAR-RPTPs themselves are involved in the presynaptic functions of mammalian neurons. To address these questions, we first monitored the paired-pulse ratio (PPR), a measure that is routinely used to identify changes in the neurotransmitter release probability (30). We first infected cultured hippocampal neurons with control lentiviruses (control) or coinfected them with lentiviruses expressing sh-LAR/sh-PTPσ and measured the PPR, which was calculated by delivering two stimuli 20 ms apart and then dividing the amplitude of the second excitatory postsynaptic current ($EPSC_2$) by the amplitude of the first EPSC ($EPSC_1$). There was no significant change in PPR between control and LAR/PTPσ-deficient neurons over a range of interstimulus intervals (20–200 ms), suggesting that LAR and PTPσ do not directly regulate the probability of neurotransmitter release at excitatory synapses (Fig. S7 *A* and *B*). We next recorded miniature ESPCs (mEPSCs) in the cultured hippocampal neurons (Fig. 5). We infected neurons with control lentiviruses expressing EGFP (control) only, sh-LAR

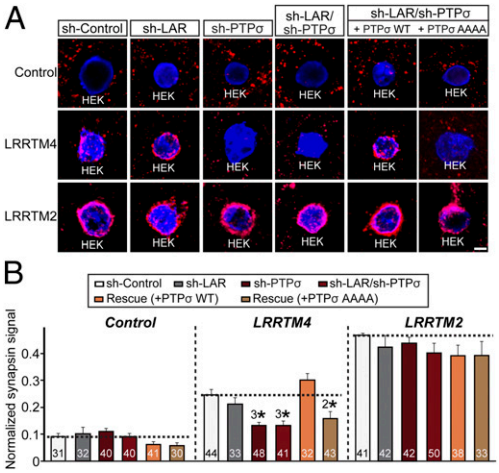

Fig. 4. PTPσ is a functional coreceptor for GPC-4 in mediating LRRTM4-induced presynaptic differentiation. (*A* and *B*) Representative images (*A*) and quantification (*B*) of the heterologous synapse-formation activities of LRRTM4 and LRRTM2. Neurons were infected with lentiviruses expressing sh-Control, -LAR, or -PTPσ only or were coinfected with lentiviruses expressing PTPσ-KD/LAR-KD (sh-LAR/sh-PTPσ), LAR/PTPσ shRNA plus PTPσ WT (+PTPσ WT rescue), or LAR/PTPσ shRNA plus PTPσ AAAA (+PTPσ AAAA rescue). The infected neurons were then cocultured for 2 d with HEK293T cells expressing EGFP alone (control), LRRTM4–EGFP (LRRTM4), or LRRTM2–mVenus (LRRTM2) and stained with antibodies against EGFP (blue) and synapsin (red). The synapse-forming activity was quantified by measuring the ratio of synapsin staining to EGFP fluorescence. The statistics shown in *D* were determined by ANOVA Tukey's test. $^{2}*P < 0.01$; $^{3}*P < 0.001$. [Scale bar: 25 μm (applies to all images).] *N* numbers are the number of HEK293T cells as indicated in the bar graphs.

only, sh-PTPσ only, sh-LAR and -PTPσ, or sh-LAR and -PTPσ with coexpression of either PTPσ WT or PTPσ–AAAA, and recorded mEPSCs (Fig. 5). Remarkably, sh-PTPσ, but not sh-LAR, significantly decreased the frequency and amplitude of mEPSCs, strengthening our previous supposition that PTPσ is required for excitatory synapse development (8). This reduction in mEPSC frequency and amplitude among PTPσ- and LAR/PTPσ-deficient neurons was completely reversed by the reexpression of PTPσ WT. Although PTPσ–AAAA rescued mEPSC amplitude, it failed to recover the mEPSC frequency (Fig. 5). To support these electrophysiological data, we used an immunocytochemical approach to determine whether PTPσ KD alters the excitatory synapse structure in cultured neurons. We found that PTPσ KD significantly decreased excitatory synapse density, as determined by VGLUT1 staining, an effect that was completely rescued by reexpression of PTPσ WT, but not PTPσ–AAAA (Fig. S7 *E* and *F*). The experimental conditions did not affect the intrinsic properties of the infected neurons, because we observed no change in the membrane capacitance (Cm) or input resistance (Rm) (Fig. S7 *C* and *D*). Our data suggest that PTPσ maintains the structure and function of excitatory synapses in a HS-binding–dependent manner.

Discussion

In the present study, we explored the significance of interactions between LAR-RPTP and GPC synaptic proteins in mammalian neurons. It is likely that these interactions are dictated by the availability of HS in neuronal membranes (Fig. 3), but we clearly demonstrated that the HS-binding activity of PTPσ is required for these proteins to act as the presynaptic receptor for a postsynaptic adhesion molecule LRRTM4 and as a key element in excitatory synaptic transmission (Figs. 4 and 5). An unusually large number (>25) of different HSPGs (31) that are expressed in the nervous system have been implicated in many aspects of neural development, including neurogenesis, axon elongation and pathfinding, and synapse formation (32). Most notably, agrins identified in neuromuscular synapses have been demonstrated to mediate acetylcholine receptor clustering (33). In addition to agrins, other neural proteoglycans, such as aggrecan, neurocan, brevican, testicans, SDCs, and GPCs, have been identified and shown to function in the nervous system (32). In recent years, SDCs and GPCs in particular have emerged as crucial regulators of cell migration and axon guidance in flies through binding to LAR (14, 28). However, their roles in mammalian synapses have only begun to be elucidated (19, 20, 34). In the present study, we found that mammalian GPCs, but not SDCs, bind to mammalian LAR-RPTPs (Fig. S5*A*). This is in stark contrast to the prevailing concept in flies that both Dally-like and SDCs bind to dLAR, suggesting that mammals and flies use a different set of ligand–receptor complexes to regulate nervous system development. Moreover, LRRTM4 exists only in vertebrates and not in invertebrates, strongly supporting the interpretation that mammals use a unique synaptic adhesion complex. Recently, GPC-4 and -6, as astrocyte-secreted signaling molecules, were demonstrated to promote excitatory synapse formation through recruitment of the AMPA receptor subunit, GluA1 (34). It is possible that a PTPσ/GPCs/LRRTM4 complex might control AMPA receptor function. In support of this supposition, LRRTM4 was recently annotated as an AMPA receptor constituent in a multiepitope proteomic analysis (35). Moreover, presynaptic neurexin-3 was recently reported to control postsynaptic AMPA receptor trafficking through direct binding to LRRTM2 (36); thus, it is probable that presynaptic PTPσ also does so by behaving similarly. Directly exploring the function of PTPσ/GPCs/LRRTM4 synaptic adhesion pathways in vivo will ultimately require systematic characterization of conditional knockout mice lacking PTPσ and/or GPCs. However, the involvement of other GPC isoforms in brain functions, particularly synapse development, has not been extensively explored. We examined the expression patterns of mRNAs encoding all six GPCs during several different developmental stages in mice (Fig. S8). We found that GPC-1 and -4 are the major isoforms in the hippocampus (Fig. S8). Intriguingly, GPC-4 mRNA was found in the dentate gyrus, whereas GPC-1 mRNA signals were stronger in the CA3 region of hippocampus, suggesting distinct distribution patterns of individual GPC isoforms in several brain regions (Fig. S8). The functional regulation of synaptic adhesion proteins by carbohydrate molecules, as reported in this work, is not unprecedented. For example, posttranslational modification of neural cell adhesion molecule (NCAM) by the addition of polysialic acid (PSA) to the fifth Ig domain

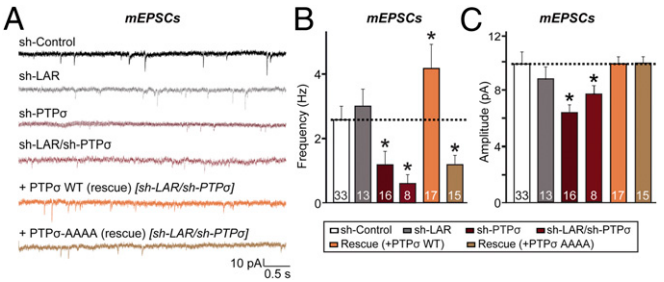

Fig. 5. PTPσ is required for HS-binding–dependent excitatory synaptic transmission in cultured hippocampal neurons. (*A*) Representative traces of mEPSCs recorded at days in vitro (DIV) 14–16 in cultured hippocampal neurons infected at DIV3 with lentiviruses expressing sh-Control, -LAR, or -PTPσ only or were coinfected with lentiviruses expressing sh-LAR/sh-PTPσ, sh-LAR/sh-PTPσ plus PTPσ WT (+PTPσ WT rescue), or sh-LAR/sh-PTPσ plus PTPσ AAAA (+PTPσ AAAA rescue). (*B* and *C*) Summary graphs of the frequencies (*B*) and amplitudes (*C*) of mEPSCs from the infected neurons. The data shown in *B* and *C* are presented as means ± SEM. $*P < 0.05$ (Student's *t* test). *N* values indicated in bar graphs correspond to the number of neurons.

abrogates the homophilic binding properties of the protein and reduces cell migration and invasion. Also, removal of PSA from NCAM by enzymatic digestion has been shown to abolish synaptic plasticity (37), suggesting that structural alterations produced by posttranslational modification of synaptic adhesion molecules is crucial for synaptic function. The importance of HS has been directly shown in knockout mice deficient for HS synthesis; these mice exhibit malformations in specific regions of the brain that reflect an altered fibroblast growth factor distribution, decreased cell proliferation, and disrupted axon pathfinding (38). Moreover, multiple signaling pathways involved in axon guidance, such as Slit–Robo and Netrin–Frazzled/DCC (deleted in colorectal cancer), require HS (39). In addition, synaptic transmission at the fly neuromuscular junction is differentially affected by KD of two different enzymes that regulate HSPG sulfation (40), suggesting that HS modifications are important for synapse development. Our findings suggest that the ability of PTPσ to bind to HS initiates multifaceted downstream signaling pathways in presynaptic neurons to control distinct aspects of excitatory synapse development. One major remaining question is how HS-bound PTPσ contributes to organizing general synapse development. HS and its analogs reportedly induce the oligomerization of PTPσ in solution, stabilize PTPσ oligomers via sulfation, and promote neurite extension (23). These findings, together with our present work, suggest that HS-bound presynaptic PTPσ induces formation of multimeric complexes with various postsynaptic ligands, thereby contributing to dynamic modulation of presynaptic organization. In addition, HS-bound PTPσ may preferentially elicit activation of specific synaptic adhesion pathway(s) via distinct *cis*- and/or *trans*-synaptic ligands.

Methods

Expression constructs and antibodies used in this study are described in detail in *SI Methods*. All cell biological assays were performed as described (27, 41). Generation of lentiviral shRNA plasmids, production, and characterization of recombinant lentiviruses were performed as described (42) and are detailed in *SI Methods*. Electrophysiology recordings in cultured hippocampal neurons were performed as described (43). See *SI Methods* for more details.

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