In situ screening for postsynaptic cell adhesion molecules during synapse formation

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Neuronal synapse formation is one of the key steps in the development of neural networks, is triggered by the trans-synaptic interaction between pre- and postsynaptic cell adhesion molecules called synaptic organizers including presynaptic neuromins (NRXN1-3) and type IIA receptor protein tyrosine phosphatases (RPTPs), and postsynaptic neuroligins (NLGN1-4), glutamate receptor GluR2/GluD2, interleukin-1 receptor accessory protein-like 1; LNS, laminin-neuroligin-exin-hormone-binding globular; NLGN, neuroligin; NMDA, N-methyl-D-aspartate; NRXN, neurexin; PDZ, PSD-95/Dlg/ZO-1; PTP, protein tyrosine phosphatase; S4, splice site 4; SALM, synaptic-attachment-like molecule; Slitrk, Slit and Trk-like; SorCS2, Sortilin-related receptor CNS expressed 2.

Neuronal synapse formation is regulated by pre- and postsynaptic cell adhesion molecules. Presynaptic neuroligins (NRXNs) and receptor protein tyrosine phosphatases (RPTPs; PTPδ, PTPζ and LAR in mammals) can induce postsynaptic differentiation through the interaction with various postsynaptic cell adhesion molecules. Here, we developed a novel in situ screening method to identify postsynaptic membranous protein complexes involved in synaptogenesis. Magnetic beads coated with the extracellular domains of NRXN1β (S4) and PTPδ-A6 variants preferentially induced excitatory postsynaptic differentiation on the beads' surface when co-cultured with cortical neurons. After inducing postsynaptic sites on these beads, protein complexes including NRXN1β (S4)/PTPδ-A6 and their ligands on the neuronal membrane were chemically cross-linked and purified using a magnetic separator. Liquid chromatography-tandem mass spectrometry analysis of the complexes revealed two types of postsynaptic ligands for NRXN1β (S4) and PTPδ-A6, one has an activity to induce presynaptic differentiation in a trans manner, whereas the other has no such activity. These results suggest that synapse formation is regulated by the interplay between presynaptic NRXN1/PTPδ and their postsynaptic ligands with functionally different impacts on pre- and postsynaptic differentiation. Thus, our in situ screening method for identifying synapse-organizing complexes will help to understand the molecular basis for elaborate neuronal networks.

Abbreviations: Cbln, cerebellin precursor protein; DIV, days in vitro; DTSSP, 3,3′-dithiobis(sulfosuccinimidylpropionate); ECD, extracellular domain; Ig, immunoglobulin-like; IL1RAPL1, interleukin-1 receptor accessory protein-like 1; LNS, laminin-neuroligin-exin-hormone-binding globular; NLGN, neuroligin; NMDA, N-methyl-D-aspartate; NRXN, neurexin; PDZ, PSD-95/Dlg/ZO-1; PTP, protein tyrosine phosphatase; S4, splice site 4; SALM, synaptic-attachment-like molecule; Slitrk, Slit and Trk-like; SorCS2, Sortilin-related receptor CNS expressed 2.
through various postsynaptic cell adhesion molecules including NLGNs, GluD2-cerebellin precursor protein (Cbln) 1 complex and leucine-rich repeat transmembrane neuronal proteins for NRXNs, and IL1RAPL1. Slitrks, synaptic adhesion-like molecules (SALMs) for type Ila RPTPs (1–4, 10–12). In mammals, NRXNs are encoded by three genes. Two alternative promoters in each gene yield a long z-NRXN and short β-NRXN (13). Type Ila RPTPs consist of three family genes products; PTPβ, PTPσ and LAR (4, 10, 11). The extracellular domain (ECD) of β-NRXN consists of a short β-NRXN-specific sequence, a single laminin–neurexin–sex hormone-binding globular (LNS) domain and the O-linked glycosylated stretch with two splice sites (14, 15). The first splice site, splice site 4 (S4), is located in the LNS domain. The second splice site is between the O-linked glycosylated stretch and the transmembrane region (14–16). The ECD of type Ila RPTPs commonly consists of three immunoglobulin-like (Ig) domains and 4–8 fibronectin type-III domains with two splice sites within Ig domains (17). The first splice site, site A, is located within the second Ig domain, and the second one, site B, is located at the junction between the second and the third Ig domains. PTPβ exists in at least eight splice variants lacking or containing short-peptide inserts, a three-residue peptide (A3), a six-residue peptide (A6) and their tandem combination (A9) at site A and a four-residue peptide at site B (17). Previously, we identified synapse-organizing complexes selectively formed between splice variant of NRXNs carrying the splice insert 4 and GluD2-Cbln1 or GluD1-Cblns, and between splice variants of PTPβ and IL1RAPL1 or interleukin-1 receptor accessory protein (17–21). So far, several splice variants selective postsynaptic ligands for NRXNs and PTPβ have been reported (4, 10, 12, 22). Thus, the alternative splicing in the ECDs of both NRXNs and type Ila RPTPs seem to enable the binding to multiple postsynaptic ligands. Nevertheless, only limited in situ information for the interaction of these molecules was available.

Previously, we have developed an in situ screening method for finding glutamate receptor GluD2 binding partner, and identified presynaptic NRXN-secreted Cbln1 complex as a GluD2 ligand (18). Here, we attempt to apply this method to identify postsynaptic membranous proteins involved in the regulation of synapse formation. Postsynaptic specializations of cortical neurons were induced on the surface of magnetic beads coated with the ECDs of NRXN1b–S4 and PTPβ–A6 variants. Then NRXN1b–S4/PTPβ–A6 on the beads’ surface and their postsynaptic ligands on the dendritic membrane were chemically cross-linked, and purified. Liquid chromatography–tandem mass spectrometry of the purified cross-linked protein complexes successfully revealed several postsynaptic ligands for NRXN1b–S4 and PTPβ–A6; some, but not all, of these showed synaptogenic activity. Thus, multiple ligands with different properties seem to be involved in the coordination of synaptogenesis.

Materials and Methods

**Screening of proteins interacting with ECDs of NRXN1b–S4 and PTPβ–A6**

Primary cerebral neurons were prepared as previously described (17) and placed on cell culture dish coated with poly-L-lysine and laminin at the density of 1.0 × 10^6 cells/10-mm dish. Magnetic beads coated with Fe fusion proteins for NRXN1b–S4-ECD or PTPβ–A6-ECD were prepared as described previously (23). The coated magnetic beads were added to the cultured cortical neurons at days in vitro (DIV) 13 at the density of 1.2 × 10^6 beads/10-mm dish. After 24 h, cultures were washed once with D-PBS and cross-linked with 1 mM 3,3'-dithiobis(sulfosuccinimidylpropionate) (DTSSP) (Pierce). Cross-linked proteins on the beads were collected and extensively washed using a magnetic separator as previously described (15). The purified proteins were treated with dithiotheitol to cleave DTSSP, separated by SDS-PAGE. All animal procedures were approved by the Animal Care and the Use Committee of Graduate School of Medicine, the University of Tokyo (Approval #1721T062). Shinshu University (Approval #280017) and Toyama University (Approval #A2016MED-32).

**Mass spectrometry**

The excised protein bands from a silver-stained gel were reduced with dithiotheitol and alkylated with iodoacetamide. Band slices were then digested with trypsin (12μg/ml) at 37°C overnight and desalted with ZipTip C18 (Millipore). The obtained peptides were separated via nano-flow liquid chromatography (nanoLC) (Paradigm MS4, AMR) using a reverse-phase C18 column. The LC eluent was coupled to a nano ion spray source attached to an LCQ Fleet mass spectrometer (Thermo Fisher Scientific). For protein identification and semi-quantification, we used the score of the SEQUEST algorithm from BioWorks software (Thermo Fisher Scientific) (24, 25).

**Construction of expression vectors**

The cDNA coding sequence of Sortilin-related receptor CNS expressed 2 (SorCS2) was amplified by RT-PCR using mRNA prepared from ICR mouse cortex and cloned into pcDNA3.1/D/V5-His-TOPO (Invitrogen) to yield pcDNA3.1-SorCS2. The entire coding sequences of GluN1 and GluN2B lacking signal sequences were amplified by PCR using pBKSα 1 (26) and pbKSA α2 (27) and cloned into the pDisplay containing an amino-terminal Ig signal peptide followed by HA tag (Clontech) and pFLAG-CMV1 (SIGMA) containing an amino-terminal preprotrypsin signal sequence followed by FLAG tag to yield pHA-GluN1 and pFLAG-GluN2B, respectively. The entire coding sequences of mouse IL1RAPL1, Shlrk4, SALM2 and SALM4 lacking signal sequences were cloned into pFLAG-CMV1 to yield pFLAG-IL1RAPL1, pFLAG-Shlrk4, pFLAG-SALM2 and pFLAG-SALM4, respectively. The DNA fragments encoding ECDs of NRXN1b–S4 and PTPβ–A6 without signal sequences were cloned into pEB6-Igk-Fc (23) to yield pEB6-NRXN1b–S4-ECD-Fc and pEB6-PTPβ–A6-Fc, respectively. The NRXN1b–S4 cDNA used for the expression vector construction includes the splice insert 5.

**Preparation of soluble recombinant proteins**

Soluble recombinant proteins were prepared as described previously (23). In brief, pEB6-Igk-Fc, pEB6-NRXN1b–S4-ECD-Fc or pEB6-PTPβ–A6-Fc was transfected into the Freestyle 293 cells (Invitrogen). Soluble recombinant Fc fusion proteins in the medium were purified by Protein A Sepharose Fast Flow (GE Healthcare), eluted with 3 M MgCl2, and dialyzed against Hanks’ Balanced Salt Solution.

**Cell cultures and co-culture assay**

Primary cortical neuron cultures were prepared from neonatal mice at postnatal day 0 as described previously (17). Co-culture assay was performed as described previously (17). In brief, HEK293T cells transfected with respective expression vector were co-cultured with cortical neurons at DIV7. After 24 h, cells were fixed with 4% paraformaldehyde and incubated with mouse anti-Bassoon antibody (Stressgen) and rabbit anti-FLAG (SIGMA) or sheep anti-SorCS2 antibody (R&D Systems), followed by incubation with species.
specific Alexa Fluor 488- and 555-conjugated antibodies. Magnetic beads coated with NRXN1β(-S4) and PTPδ-A6 were prepared as described above. The coated beads were applied to the cultured cortical neurons at DIV13. After 24 h incubation, cells were fixed with 4% paraformaldehyde and incubated with rabbit anti-Shank2 (28) and mouse anti-gephyrin (Synaptic Systems) antibodies, followed by incubation with species specific Alexa Fluor 488- and 555-conjugated antibodies.

**Image acquisition and quantification**

Image acquisition and quantification were performed as described previously (17). In brief, the intensities of immunostaining signals for presynaptic or postsynaptic proteins were measured as the mean fluorescent intensity within circles of 30- and 7-μm diameters enclosing transfected HEK293T cells and coated-beads, respectively. Statistical significance was evaluated by Student’s t test or one-way ANOVA followed by post hoc Tukey’s test. Statistical significance was assumed when P < 0.05.

**Results**

**Screening of postsynaptic proteins interacting with ECDs of NRXN1β(-S4) and PTPδ-A6**

We first examined the activity of NRXN1β(-S4) and PTPδ-A6 to induce postsynaptic differentiation, because both splice variants are highly expressed in cortical neurons (29) (Yoshida et al., unpublished observation). Cultured cortical neurons were incubated with magnetic beads coated with an equal amount of the respective ECDs of NRXN1β(-S4) and PTPδ-A6 (Fig. 1). After incubation for 24 h, the cultures were immunostained with antibodies against excitatory and inhibitory postsynaptic scaffold proteins, Shank2 and gephyrin, respectively. We detected numerous punctate signals for Shank2 on the surface of both NRXN1β(-S4)-ECD- and PTPδ-A6-ECD -coated beads (Fig. 1A). The staining signals on the surface of these beads were significantly stronger than those on the control Fc-coated beads (P < 0.01; Fig. 1B). On the other hand, faint accumulation of gephyrin signals was detected on the surface of the NRXN1β(-S4)-ECD-coated beads (Fig. 1A). There was a significant difference in the staining signals for gephyrin between NRXN1β(-S4)-ECD- and control Fc-coated beads (Fig. 1B). These results suggest that the ECDs of NRXN1β(-S4) and PTPδ-A6 on the beads’ surface preferentially induced the excitatory postsynaptic differentiation of co-cultured cortical neurons in a trans manner through the binding partners on the dendritic membrane.

To find the trans-synaptic postsynaptic binding partners of NRXN1β(-S4) and PTPδ-A6 in the cortical neurons, postsynaptic differentiation was induced with magnetic beads conjugated with the ECD of NRXN1β(-S4) and that of PTPδ-A6, respectively (Fig. 1C). After co-culturing for 24 h, the surface proteins of the dendrites of cortical neurons were cross-linked to the ECDs of NRXN1β(-S4) and PTPδ-A6 on the magnetic beads with non-membrane-permeable chemical cross-linker DTSSP, respectively, as described previously (18). Then, the cross-linked proteins were identified by liquid chromatography-tandem mass...
respectively; Fig. 3B). Thus, we successfully identified GluN2B were incubated with the PTP IL1RAPL1, Slitrk4, SALM2, SALM4 or GluN1/GluN2B. The transfected HEK293T cells expressing IL1RAPL1, Slitrk4, SALM2, SALM4 and GluN1/GluN2B. The transfected HEK293T cells expressing IL1RAPL1, Slitrk4, SALM2, SALM4 and GluN1/GluN2B. The transfected HEK293T cells expressing IL1RAPL1, Slitrk4, SALM2, SALM4 and GluN1/GluN2B expressing HEK293T expressing cells (Fig. 2A). The staining signals on the HEK293T cells expressing NLGN1, NLGN3, or SorCS2 were significantly stronger than those cells incubated with the control Fc (P<0.001, P<0.001 and P<0.01, respectively; Fig. 2B).

Then, we examined the interaction of PTPδ-A6 with IL1RAPL1, Slitrk4, SALM2, SALM4 and GluN1/GluN2B. The transfected HEK293T cells expressing IL1RAPL1, Slitrk4, SALM2, SALM4 or GluN1/GluN2B were incubated with the PTPδ-A6-ECD fused to Fc and then stained as described above. We detected strong staining signals for the Fc fusion protein on the surface of IL1RAPL1, Slitrk4, SALM2 or SALM4 expressing HEK293T cells but not on GluN1/GluN2B expressing cells (Fig. 3A). The staining signals on the HEK293T cells expressing IL1RAPL1, Slitrk4, SALM2 or SALM4 were significantly stronger than those cells incubated with the control Fc (P<0.001, respectively; Fig. 3B). Thus, we successfully identified the trans-synaptic postsynaptic binding partners of NRXN1β(−S4) and PTPδ-A6. To the best of our knowledge, the direct interactions between NRXN1β(−S4) and SorCS2 and those between PTPδ-A6 and SALM2 or SALM4 have never been reported.

Table I. A list of membrane proteins identified from NRXN1β(-S4)-Fc and PTPδ-A6-Fc coated beads

<table>
<thead>
<tr>
<th>Bait Protein</th>
<th>Probabilitya</th>
<th>Scoreb</th>
<th>Hitsd</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRXN1β(-S4)-Fc</td>
<td>NLGN1</td>
<td>3.70E-07</td>
<td>10.15</td>
</tr>
<tr>
<td></td>
<td>GluN2B</td>
<td>3.99E-07</td>
<td>22.15</td>
</tr>
<tr>
<td></td>
<td>NLGN3</td>
<td>9.66E-07</td>
<td>38.16</td>
</tr>
<tr>
<td></td>
<td>SorCS2</td>
<td>2.74E-06</td>
<td>30.15</td>
</tr>
<tr>
<td></td>
<td>GluN1</td>
<td>5.43E-05</td>
<td>26.13</td>
</tr>
<tr>
<td></td>
<td>GluN2B</td>
<td>9.83E-08</td>
<td>52.21</td>
</tr>
<tr>
<td>PTPδ-A6-Fc</td>
<td>IL1RAPL1</td>
<td>4.66E-05</td>
<td>8.07</td>
</tr>
<tr>
<td></td>
<td>Slitrk4</td>
<td>2.21E-06</td>
<td>16.17</td>
</tr>
<tr>
<td></td>
<td>SALM2</td>
<td>7.90E-06</td>
<td>10.18</td>
</tr>
<tr>
<td></td>
<td>SALM4</td>
<td>1.11E-05</td>
<td>20.19</td>
</tr>
</tbody>
</table>

Proteinsa, proteins with probability value (<1.0E−04) are listed; Probabilityb, probability (protein) of finding a match as good as or better than the observed match by chance; Scorec, SEQUEST scores; Hitsd, number of unique parent peptides found.

question: The NRXN1β(−S4)-ECD and the PTPδ-A6-ECD bind to identified membrane proteins

We next examined the interaction of NRXN1β(−S4)-ECD with NLGN1, NLGN3, SorCS2, GluN1 and GluN2B through a cell surface binding assay. Each protein was expressed in HEK293T cells and incubated with the NRXN1β(−S4)-ECD fused to Fc followed by staining with antibody against Fc. Because functional NMDA receptor forms a heterotetramer between two GluN1 and two GluN2 subunits (27), HEK293T cells were co-expressed with both GluN1 and GluN2B for the binding assay. We detected strong staining signals for the Fc fusion protein on the surface of NLGN1, NLGN3 or SorCS2 expressing HEK293T cells but not on GluN1/GluN2B expressing cells (Fig. 2A). The staining signals on the HEK293T cells expressing NLGN1, NLGN3, or SorCS2 were significantly stronger than those cells incubated with the control Fc (P<0.001, P<0.001 and P<0.01, respectively; Fig. 2B).

Fig. 2 Interactions between NRXN1β(−S4) and proteins identified by the screening. (A) Binding of ECD of NRXN1β(−S4)-Fc or Fc to HEK293T cells expressing NLGN1, NLGN3, SorCS2 or GluN1/GluN2B. Expressed proteins (upper panels) and cell surface-bound Fc or Fc fusion proteins (lower panels) were immunostained. Scale bar represents 10 μm. (B) Ratios of staining signals for Fc or Fc fusion proteins and expressed proteins in (A) (n = 10 HEK293T cells each). All values represent mean ± SEM. **P<0.01 and ***P<0.001, respectively; t-test.

NLGN1, NLGN3, IL1RAPL1 and Slitrk4 showed presynapse-inducing activity, whereas SorCS2, SALM2 and SALM4 showed no such activity

Axo-dendritic contacts bidirectionally induce the presynaptic and postsynaptic differentiation at the contact sites (30), and the trans-synaptic interactions between NRXNs and NLGNs and between PTPδ and IL1RAPL1 are well known to bidirectionally induce synaptic differentiation (17, 31–32). Thus, by using HEK293T cell-neuron co-cultures, we examined whether the identified postsynaptic ligands for NRXN1β(−S4) and PTPδ-A6 show presynapse-inducing activity or not. The HEK293T cells expressing NLGN1, NLGN3, SorCS2, IL1RAPL1, Slitrk4, SALM2 or SALM4 were co-cultured with cultured cortical neurons. After co-culturing for 24 h, the cells...
were fixed and stained with antibody against the presynaptic active zone protein Bassoon. We detected the accumulation of Bassoon signals on the surface of NLGN1, NLGN3, IL1RAPL1 or Slitrk4 expressing HEK293T cells (Fig. 4A). The staining signals for Bassoon on the transfected HEK293T cells were significantly stronger than those on HEK293T cells expressing EGFP (P < 0.01; Fig. 4B). In contrast, the staining signals for Bassoon on HEK293T cells expressing SorCS2, SALM2 or SALM4 were comparable to those on HEK293T cells expressing EGFP (Fig. 4B), indicating that these ligands have no presynapse-inducing activity.

Discussion

Synapse formation is highly spatiotemporally organized by multiple and diverse synaptic cell adhesion molecules. Much evidence has been presented for cell-type-, region-specific or biased expression of synaptic cell adhesion molecules in the brain (12). Thus, certain cell-type- or region-specific trans-synaptic cell adhesion complexes must be elucidated for understanding of wiring specificity and synaptic diversity. Herein, we developed a novel screening method to identify postsynaptic membranous proteins involved in situ in synaptogenesis.

In this study, by using artificially induced hemi-synapses between recombinant synapse organizer-coated beads and cultured cortical neurons, we identified NLGN1, NLGN3 and SorCS2 as postsynaptic ligands of presynaptic NRXN1β (−S4) as well as IL1RAPL1, Slitrk4, SALM2 and SALM4 as postsynaptic ligands of presynaptic PTPδ-A6. Postsynaptic NLGN1 and NLGN3 are reported as NRXN ligands, and...
Physically close at these synapse organizers. A previous (Table I), indicating that NMDA-type receptors are ECD- and PTP

interaction. Interestingly, GluN1 and/or GluN2B protein will be required to understand the functions of this and SorCS2 remained unclear. Further investigation (Table II) of neuronal viability and functions through inter-

actions with neurotrophic factors and their receptors of neuronal networks in the brain. Acknowledgements

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**References**


