Supporting Information for

Facile synthesis of dimeric thioether-macrocyclic peptides with antibody-like affinity against Plexin-B1

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Materials

All standard Fmoc amino acids as well as Fmoc-NH-PEG_1-COOH, Fmoc-NH-PEG_5-COOH, Fmoc-NH-PEG_{11}-COOH, Novapeg Rink Amide resin and HBTU were purchased from Merck Millipore. Fmoc-2,3-diaminopropionic acid(Fmoc), Fmoc-β-Alanine and HOBt were purchased from Watanabe Chemical Industries, Ltd.. DMF, NMP, DMSO, Acetonitrile, DIPEA, Piperidine, TFA, TIS and EDT were purchased from Nacalai Tesque. All peptide synthesis was performed on a Syro Wave automated peptide synthesizer (Biotage). All purification was performed on a Prominence RP-HPLC system (Shimadzu).

Methods

Synthesis of peptides in this study.

Monomeric peptide PB1m6 was synthesized as described previously (Matsunaga et al, 2016). All dimeric peptides were synthesized using conventional Fmoc-based SPPS on a Syro I automated peptide synthesizer (Biotage). Coupling of the initial Fmoc-DAP(Fmoc), Fmoc-NH-PEG_X-COOH linker and other Fmoc-protected amino acids were performed on DMF engorged NovaPEG Rink Amide resins with 0.5 M Fmoc-protected amino acid, 0.5 M HBTU/HOBt and 0.5 M DIPEA in DMF. After three programmed washes with DMF, Fmoc-deprotection was performed by a 30 min RT treatment with 40% piperidine in DMF. Double coupling was used for all steps. After all amino acids were coupled, 0.2 M chloroacetyl-NHS in DMF was used to add a chloroacetyl group to the N-terminus of each peptide. This step was repeated twice, followed by three DMF washes, six DCM washes and desiccation in vacuo. Dessicated peptide-conjugated resins were then globally deprotected and cleaved from the resin in a mixture of pre-chilled TFA/H_2O/EDT/TIS (92.5 : 2.5 : 2.5 : 2.5) for 1 hr at RT. The deprotection solutions were precipitated with chilled diethyl ether and the pellets were dried in fume hoods. These pellets were dissolved in DMSO 0.1% TFA followed by the addition of TEA until the solution was basic (confirmed using H_2O and pH indicator paper) to induce cyclization of the peptides. After 30 min at RT in basic conditions, peptide mass and cyclization was confirmed by MALDI-TOF MS analysis (Autoflex II Bruker Daltonics). Once peptide mass and cyclization was confirmed, the peptide solution was brought back to acidic conditions with TFA (confirmed using H_2O and pH indicator paper) and purified on a Shimadzu Prominence RP-HPLC using a Chromolith ODC column (Flow rate: 25 mL/min. Gradient: 10 - 70 % MeCN (containing 0.1 % TFA) in deionized H2O (containing 0.1 % TFA) over 32 min.

MALDI-TOF MS analysis

All MALDI-TOF MS analysis was performed on an UltrafleXtreme MALDI-TOF/TOF MS (Bruker). Dimeric peptides dissolved in 80% acetonitrile aq. were spotted on a Ground Steel Plate 384 Target plate 1:1 with a saturated solution of sinapinic acid (Bruker) in 80% acetonitrile 0.5% acetic acid aq and ionized on linear-positive mode.

Ultra high performance liquid chromatography (UHPLC)
The purity of synthesized peptides were analyzed on a SHIMAZU NexeraX with an ACQUITY UPLC Protein BEH C18 column.

Specific conditions for "FigureS1. UHPLC analysis of PB1m6 cyclization at various concentrations":
Sample: 2. 5 μL of 50 μM peptide solutions in 50% DMSO, 25% MeCN, 25% H2O 0.1% TFA.
Column temperature: 40°C
Analysis time: 10 min
Flow rate: 0.7 mL/min
Gradient: 35- 40 % MeCN (containing 0.1 % TFA) in deionized H2O (containing 0.1 % TFA)

Specific conditions for "FigureS3. UHPLC chromatograms of crude PB1m6 and PB1d6P10":
Sample: 5 μL of crude peptide solution in 50% DMSO, 25% MeCN, 25% H2O 0.1% TFA.
Column temperature: 40°C
Analysis time: 10 min
Flow rate: 0.7 mL/min
Gradient: 10 -70 % MeCN (containing 0.1 % TFA) in deionized H2O (containing 0.1 % TFA)

Specific conditions for "FigureS5. UHPLC of purified peptides":
Sample: 2.5 μL of 50 uM peptide solution in 50% DMSO, 25% MeCN, 25% H2O 0.1% TFA.
Column temperature: 40°C
Analysis time: 10 min
Flow rate: 0.7 mL/min
Gradient: 10 -70 % MeCN (containing 0.1 % TFA) in deionized H2O (containing 0.1 % TFA)
Purity (peptide peak area*100/total peak area) was calculated by SHIMAZU, LabSolutions

Surface plasmon resonance analysis (SPR)
Binding kinetics and dissociation constants were analyzed on a Biacore T200 (GE Healthcare). An anti-PA tag antibody was immobilized via amine coupling on a CM5 sensor chip. PA-tagged hPlexin-$B1_{SP}$ were immobilized to capture approximately 600 RUs. PB1m6 was injected as analyte at 50, 25, 12.5, 6.25 and 3.13 nM and all dimeric peptides were injected at 10, 5, 2.5, 1.25, 0.63 nM concentrations. Association times were set to 120 s and dissocation times were set to 600 s. All analyses were performed in TBST 0.1% DMSO at 25ºC.

SEAP-ligand binding assay
SEAP-ligand binding assay was performed by a method described previously (Berger et al., 1998), with a slight modification. A DNA fragment corresponding to the ectodomain of human Sema4D (residues 22-730) was amplified, fused to the C-terminus of human Alkaline Phosphatase (AP) (residues 18-507), and cloned into a pcDNA3.1 vector. The resultant plasmid coding for AP-hSema4Decto was transfected into HEK293T cells, and the culture supernatant was collected at 4 days post transfection. The conditioned media containing AP-hSema4Decto was added to PA-tagged hPlxnB1sp immobilized on anti-PA-tag NZ-1 antibody conjugated agarose beads, and incubated for 24 hours at 4 ºC. The beads were incubated in the presence of various concentrations of the macrocyclic peptides. After three washes with PBS (10 mM phosphate, pH 7.4, 150 mM NaCl), the bead-immobilized hPlnxB1sp and the hPlxnB1-bound AP-hSema4Decto were eluted from the beads with a solution containing 0.3 mg/mL free PA-tag peptide. The amount of AP-hSema4Decto associated with the beads was quantitated by measuring the AP enzymatic activity in the peptide-eluted samples using a chromogenic phosphate substrate *p*-nitrophenyl phosphate (Sigma).
See also: Berger J.; Hauber J.; Hauber R.; Geiger R.; Cullen BR. *Gene* **1988** Jun 15;66(1): 1-10.

Cellular collapse assay

The hPlxnB1 stably expressing cell line was established by using Expi293F cells as previously described (Matsunaga et al., 2016). The cells (2×10^4 cells/well) were seeded onto 10 × 10 mm glass-bottom coverslips coated with 4 μg/cm^2 fibronectin. After 24 hours, the cells were treated with PB1m6 or PB1d6P10 for 15 min at 37 ºC, and then incubated with hSema4D-Fc for 60 min at 37 ºC. All incubations were conducted in serum-free DMEM. After fixing the samples with 4% paraformaldehyde in PBS for 30 min at room temperature, the nuclei were stained with Hoechst 33342 (Invitrogen) for 4 hours at 4 ºC with protection from light for cell counting.

See also: Matsunaga, Y.; Bashiruddin, N. K.; Kitago, Y.; Takagi, J.; Suga, H. *Cell Chem. Biol.* **2016**, *23* (11), 1341.

3D-Structural models

The 3D structural models in this study were made using Maestro version 11.1.012. The 3D structures of peptide PB1m6, Plexin-B1 and Semaphorin-4D were rendered based on structures from PDB IDL: 5B4W and 3OL2. The linker segments were rendered using MacroModel (version 10.7 Schrodinger, Inc.) with OPLS3 as force field.

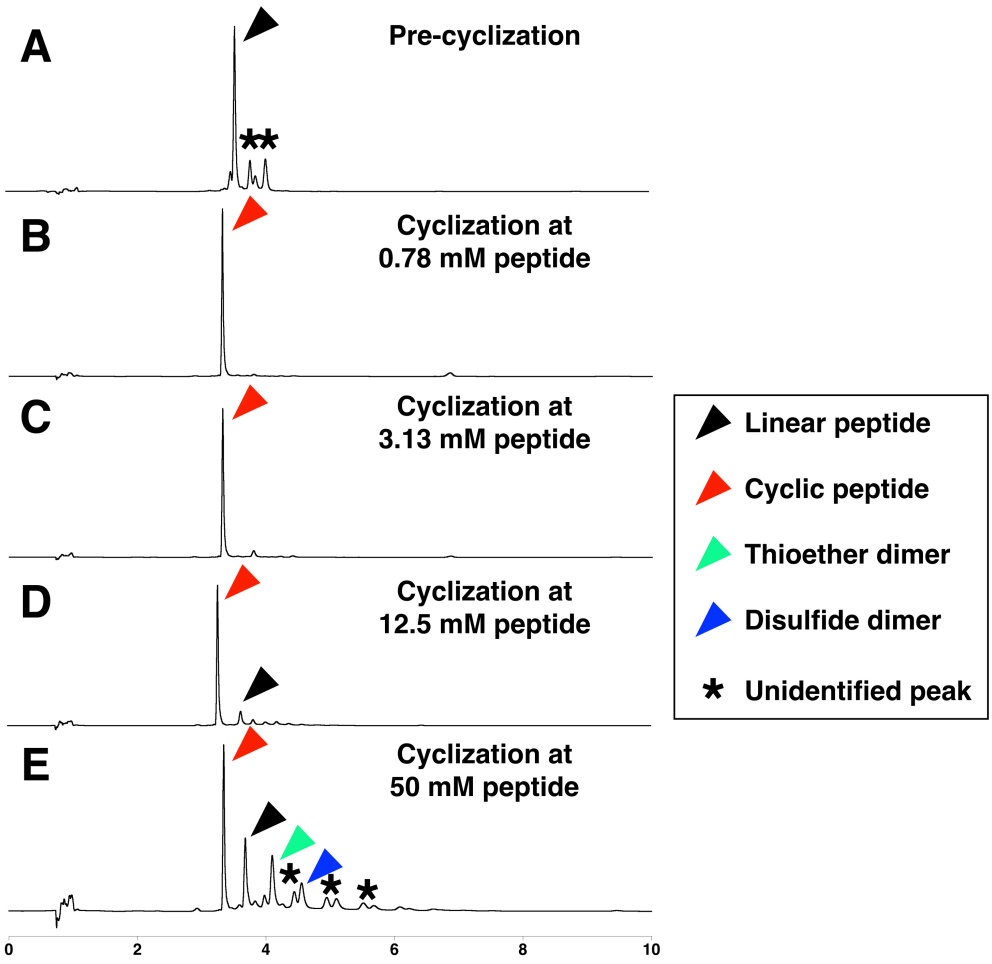


Figure S1. UHPLC analysis of PB1m6 cyclization at various concentrations. (A) PB1m6 was purified in linear form before cyclization which leaves an N-terminal chloroacetyl group. (B-E) Linear PB1m6 was diluted to denoted concentrations and brought to basic conditions with triethylamine for 30 minutes to observe possible intermolecular thioether or disulfide formation compared to intramolecular thioether formation.

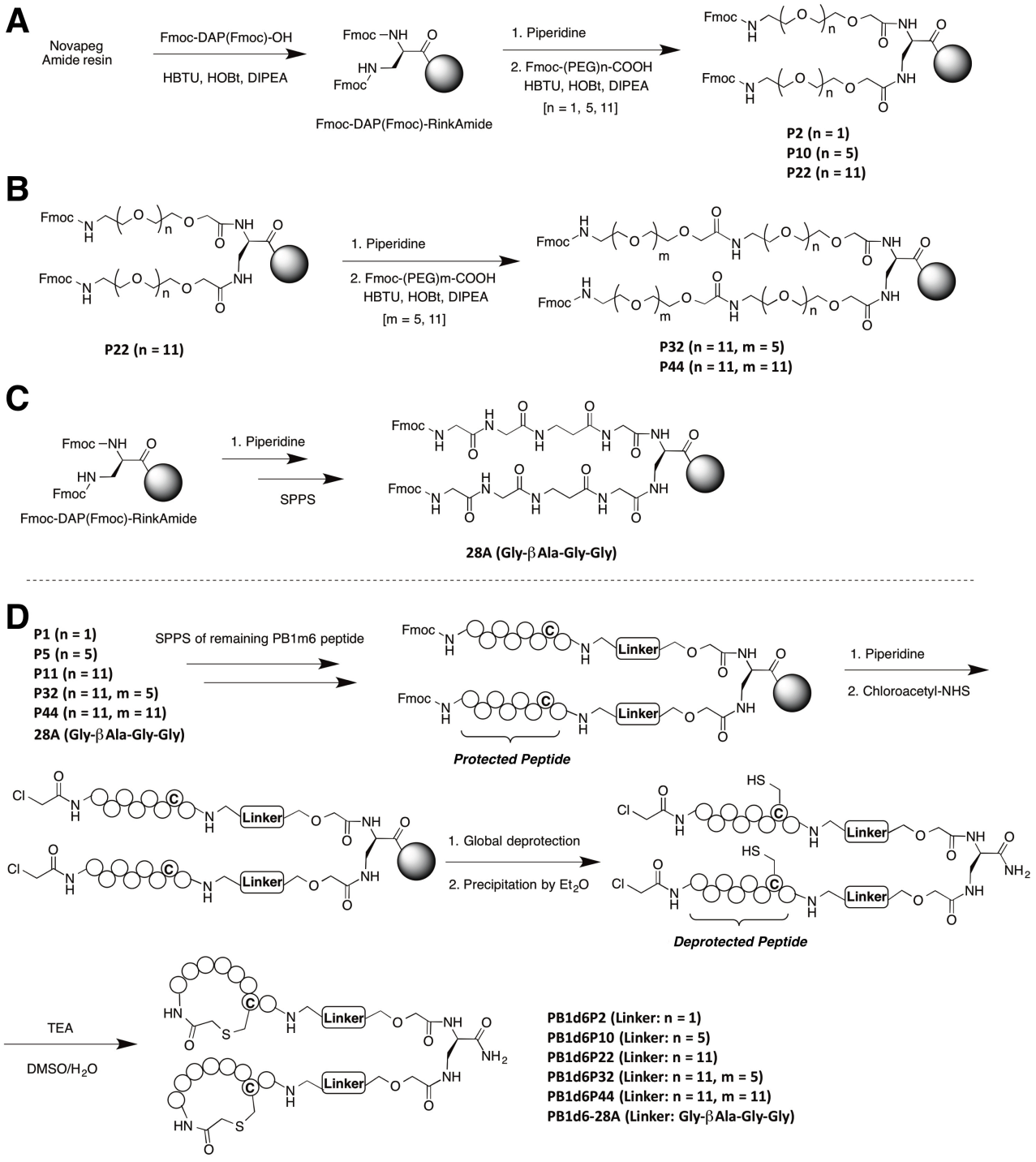


Figure S2. Synthesis scheme for dimeric cyclic peptides. (A) Solid phase syntheis of the dimer node and linkers for PB1d6P2, PB1d6P10 and PB1d6P22. (B) Synthesis of the dimer node and linkers for PB1d6P32 and PB1d6P44 starting with the dimer node and linker of PB1d6P22. (C) Solid phase synthesis of the dimer node and linker of PB1d6-28A. (D). Synthesis of the macrocyclic peptide regions of all dimeric cyclic peptides.

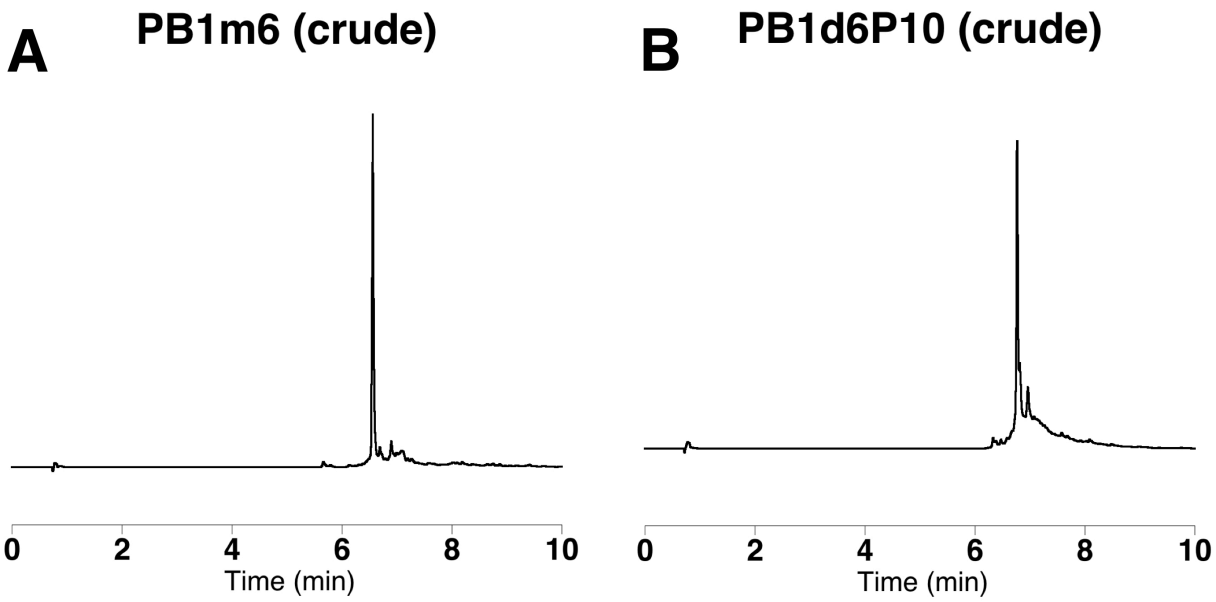
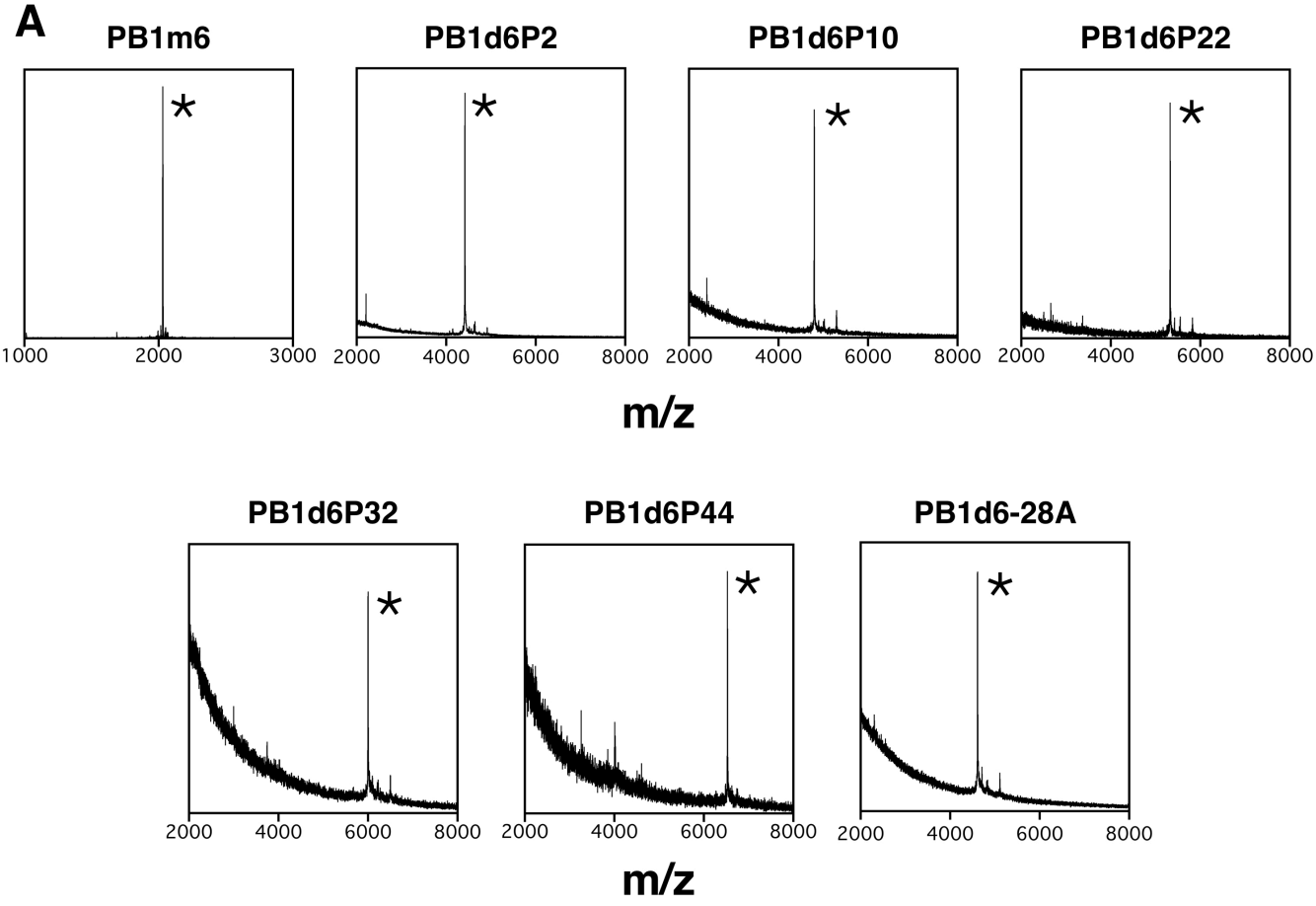


Figure S3. UHPLC chromatograms of crude PB1m6 and PB1d6P10. Crude samples of (A) PB1m6 and (B) PB1d6P10 were analyzed by UHPLC. Both (A) and (B) were synthesized in parallel with subsequent deprotection, ether precipitation and cyclization steps performed under the same conditions.

A

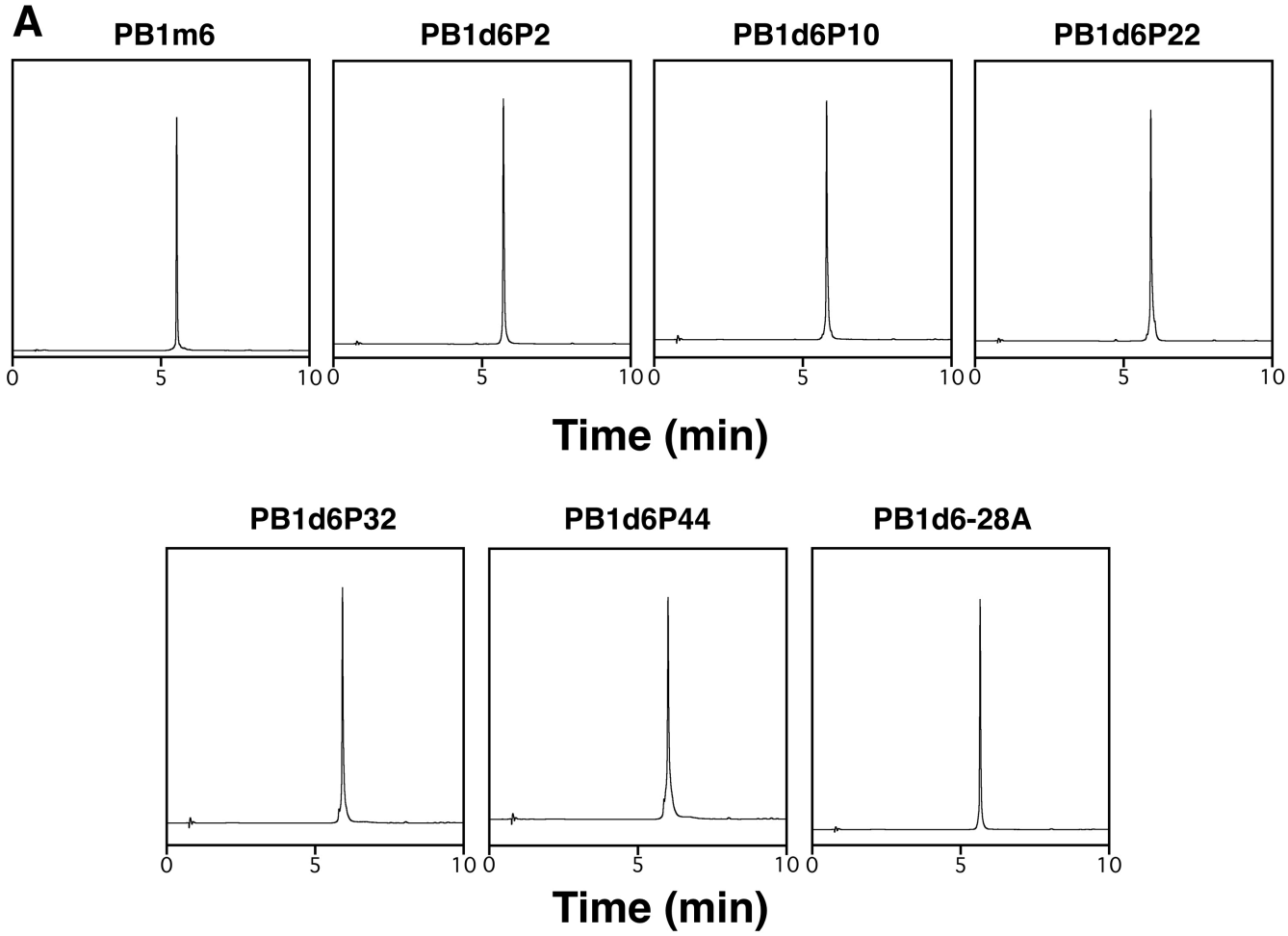


B

	Calc [M+H]	*Obs [M+H]	Abs. Difference
PB1m6	2030.04	2030.84	0.8

	Calc [MW+H]	*Obs [MW+H]	Abs. Difference
PB1d6P2	4423.14	4420.93	2.21
PB1d6P10	4803.62	4802.07	1.55
PB1d6P22	5332.26	5329.58	2.68
PB1d6P32	6003.05	6003.8	0.75
PB1d6P44	6531.69	6529.48	2.21
PB1d6-28A	4617.3	4615.27	2.03

Figure S4. MALDI-TOF MS analysis of PB1m6 and dimeric cyclic peptides. (A) Mass spectra of PB1m6 and dimeric cyclic peptides. (B) Calculated and observed exact mass + proton ([M+H]) or molecular weight + proton ([MW+H]) values for main peaks in (A) and the absolute mass differences in the far-right column.

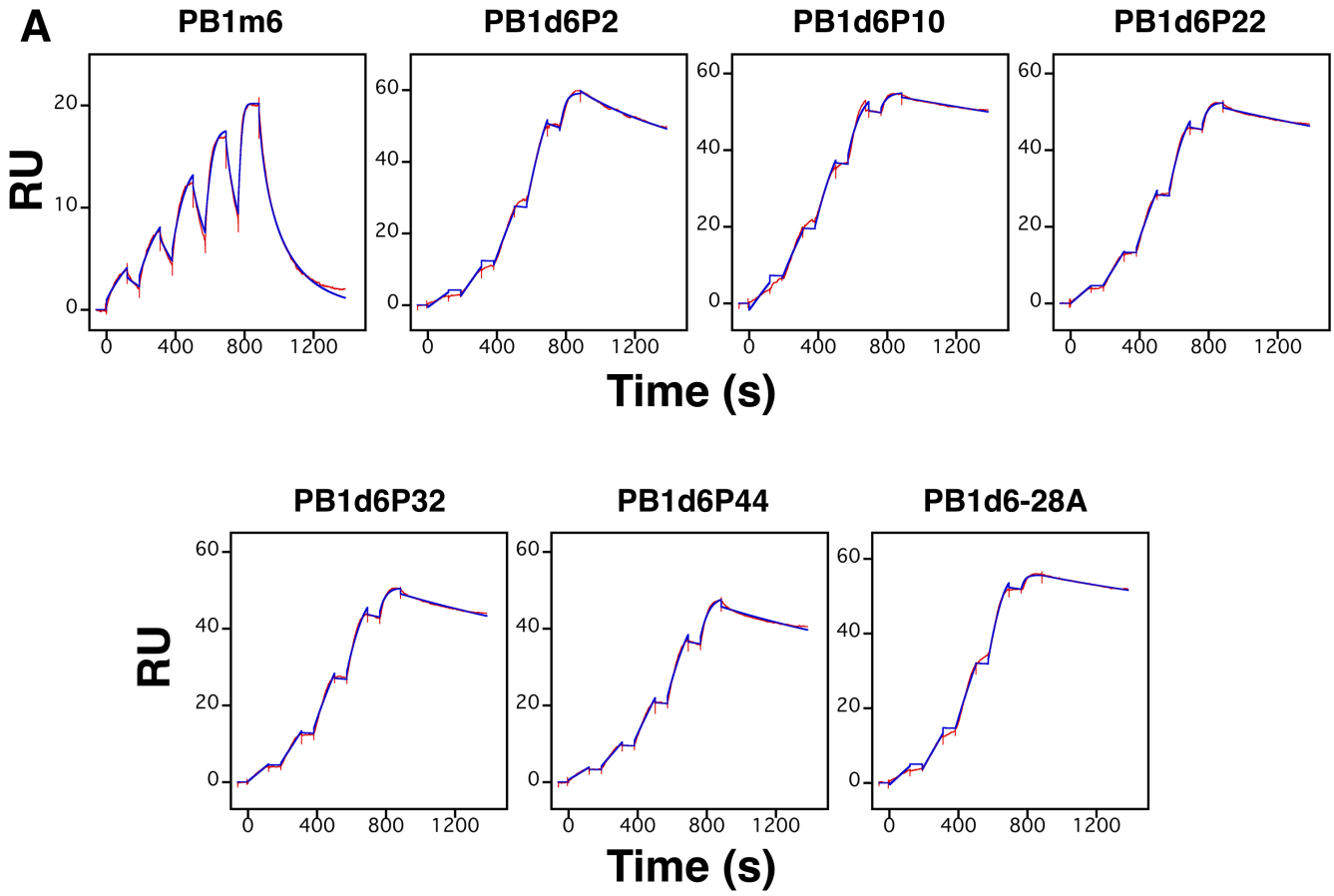


B

	t_R (min)	Purity (%)
PB1m6	5.534	96.9
PB1d6P2	5.724	98.4
PB1d6P10	5.805	95.3
PB1d6P22	5.918	96.5
PB1d6P32	5.926	89.3
PB1d6P44	6.018	94.2
PB1d6-28A	5.661	96.1

Figure S5. UHPLC of purified peptides. Upon completion of synthesis, the major product from each synthesis was collected through preparative HPLC and reanalyzed via UHPLC to confirm a homogenous species. (A) UV (280 nm) spectra from UHPLC analysis of PB1m6 and dimeric peptides. (B) Retention times (t_R) and purities of PB1m6 and dimeric peptides from (A).

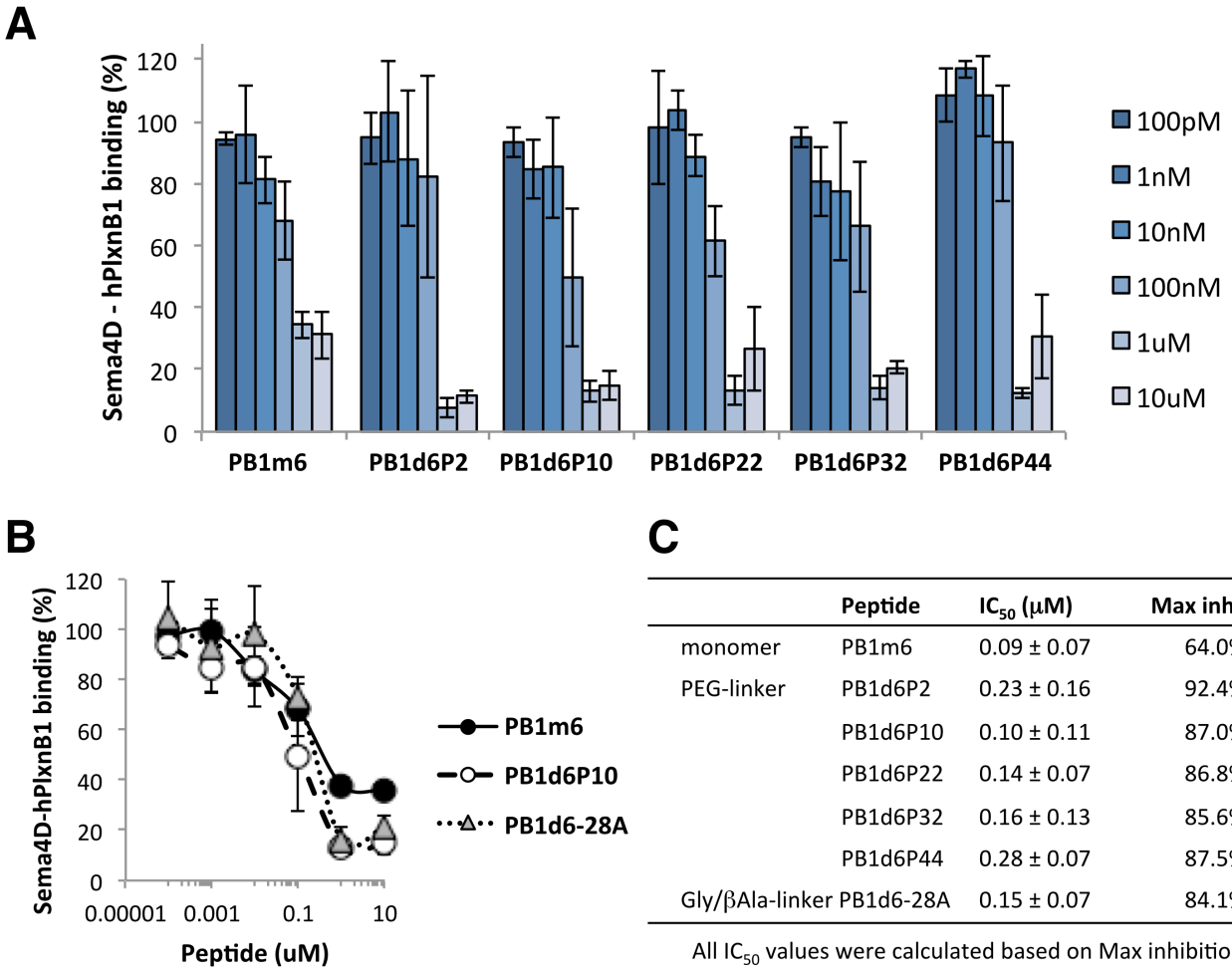
A



B

	ka (1/Ms)	kd (1/s)	KD (M)
PB1m6	2.46E+06	2.11E-02	8.59E-09
PB1d6P2	5.19E+06	5.67E-04	1.09E-10
PB1d6P10	2.96E+06	1.35E-04	4.56E-11
PB1dP22	3.77E+06	2.18E-04	5.77E-11
PB1d6P32	3.47E+06	2.82E-04	8.11E-11
PB1d6P44	2.81E+06	3.33E-04	1.19E-10
PB1d6-28A	5.67E+06	1.70E-04	3.01E-11

Figure S6. Surface plasmon resonance (SPR) analysis of PB1m6 and dimeric peptides. (A) SPR sensorgrams of PB1m6 and dimeric cyclic peptides (Red lines = response, Blue lines = fitted). (B) Binding kinetics and dissociation constants (K_D) from (A).



	Peptide	IC_{50} (μM)	Max inhibition
monomer	PB1m6	0.09 ± 0.07	64.0%
PEG-linker	PB1d6P2	0.23 ± 0.16	92.4%
	PB1d6P10	0.10 ± 0.11	87.0%
	PB1d6P22	0.14 ± 0.07	86.8%
	PB1d6P32	0.16 ± 0.13	85.6%
	PB1d6P44	0.28 ± 0.07	87.5%
Gly/βAla-linker	PB1d6-28A	0.15 ± 0.07	84.1%

All IC_{50} values were calculated based on Max inhibition

Figure S7. SEAP-ligand binding experiments to assay for Semaphorin4D-PlexinB1 protein-protein interaction inhibitory activity of PB1m6 and dimeric cyclic peptides. (A) Results from SEAP-ligand binding assays from PB1m6, PB1d6P2, PB1d6P10, PB1d6P22, PB1d6P32 and PB1d6P44. Histograms represent the degree of Semaphorin4D-PlexinB1 binding. (B) Scatter-plot of SEAP-ligand binding experiments comparing PB1m6, PB1dP10 and PB1d6-28A. (C) Summary table of results from (A-B). All data are shown as means ± SD from three independent analyses

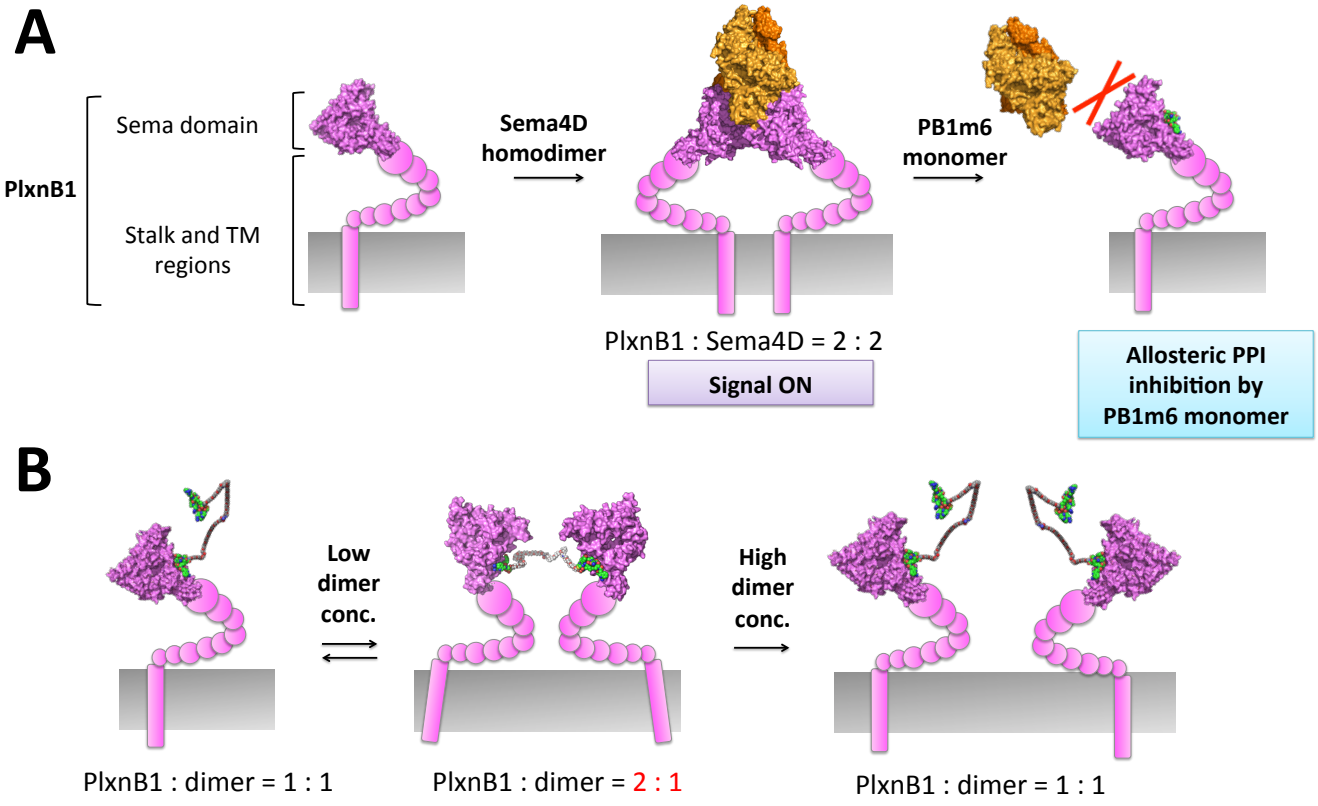


Figure S8. Breakdown schematic representation of the hypothetical inhibitory mechanisms of PB1m6 and PB1m6-based dimers. (A) PB1m6 (green) has been previously shown (Matsunaga et al. 2016) to allosterically inhibit the interaction between PlexinB1 (PlxnB1, magenta) and Semaphorin4D (Sema4D, orange) therefore inhibiting Sema4D-dependent PlxnB1 dimerization and concomitant downstream signaling. (B) At low concentrations of PB1m6-dimers, PB1m6-dimers and PlxnB1 exist in either 1:1 or 1:2 (incorrectly oriented dimer) complexes while at high PB1m6-dimer concentrations the 1:1 complex predominates (same inhibition mode as monomer but stronger due to higher binding affinity).