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A NEW APPLICATION OF A PEPTIDE LIBRARY TO IDENTIFY SELECTIVE INTERACTION BETWEEN SMALL PEPTIDES IN AN ATTEMPT TO DEVELOP RECOGNITION MOLECULES TOWARD PROTEIN SURFACES

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Abstract: Combinatorial technologies with the use of a solid-phase pentapeptide library and the magnetic beads covalently bound with the target nonapeptide (**1**) were applied to search for recognition peptides toward the target sequence (**1**) which constitutes an extracelluar part of the dopamine D2 receptor. Several pentapeptides have been identified to exhibit high binding affinities toward the target peptide (**1**) in an aqueous solution.

Selective interactions between small peptide fragments have attracted much attention from the viewpoint of several aspects such as the determining factors of protein conformation and the origin of specific interactions between proteins or receptor-ligands, *etc*. We anticipate that small peptides which can recognize particular parts of proteins will be useful to discriminate a target protein from diverse subtypes, and that such recognition molecules will be developed based on selective interactions between the small peptides. Since a rational design of the recognition molecules based on a peptide sequence or even on 3D structures seems to be difficult because of the complex nature of the recognition modes of peptides,[1, 2] it is desirable to develop a convenient method to search for small peptides selective toward the target sequence.[3] Here, we wish to report, in our attempts to find recognition molecules for the dopamine D2 receptors, that we have determined selective peptides toward the target peptide sequence by a random search with combinatorial technologies using the "one-sequence-per-one-bead" pentapeptide library[4] and the magnetic beads covalently bound with the target peptide. Although peptide libraries have been extensively utilized to identify peptide ligands to receptors, this study has demonstrated the first example where small peptides have been identified as recognition molecules to the target small peptide.

In our search for the recognition molecules for the CNS dopamine D2 receptors, the extracellular nonapeptide sequence (residues 7-15: Ser-Trp-Tyr-Asp-Asp-Asp-Leu-Glu-Arg (**1**)) was chosen as the target

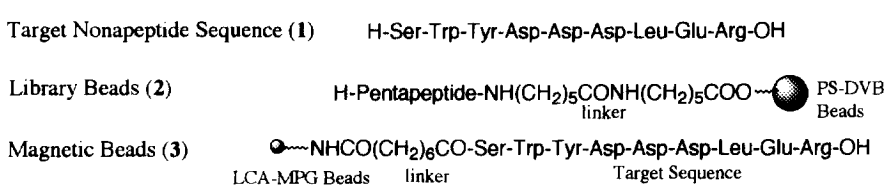

Fig. 1. The target peptide (**1**), pentapeptide library (**2**) and magnetic beads (**3**).

peptide, because this region is known to characterize the receptor subtype.[5] The pentapeptide library (**2**) was constructed using *N*-Fmoc derivatives of 20 natural L-amino acids onto polystyrene-divinyl benzene beads[6] according to the "one-sequence-per-one-bead" concept reported by K. S. Lam *et al*;[4] thus, the library contains 20^5 peptide sequences.[7] For easy detection of the interaction between library beads and the target peptide, the target nonapeptide (**1**) was covalently introduced onto the magnetic beads (**3**).[7,8] As magnetic beads have been used to isolate selective ligands for the molecule that is introduced onto the beads,[9] we applied this method to select library beads bound with the magnetic beads. Peptides were introduced onto each bead with a long linker to avoid nonspecific bead-bead interaction (Fig. 1).

The library (**2**) and the magnetic beads (**3**)[10] were mixed in an aqueous PBS buffer at pH 6.8[11] in a test tube, and gently shaken for two hours. Then, the test tube was allowed to stand in contact with an outer magnet until there was no further sedimentation. The library-magnetic bead complexes adhered to the glass surface where the magnet was attached outside, and were collected as the selective beads. These bead-bead complexes were formed apparently based on peptide-peptide interactions, because magnetic beads without the target nonapeptide did not form complexes with the library beads. Bead-bead complexes which were bound by strong ionic interaction were removed by the same selective method in an aqueous buffer at pH 7.9. Finally, six sequences were determined (**4-9**) from millions of the library beads. To estimate interactions between the selected (**4-9**) and the target peptide (**1**), standard titration experiments were monitored by both UV and ^{1}H-NMR spectroscopy in an aqueous buffer at pH 6.8.[11] When a solution of the pentapeptide[12] was added to a solution of the nonapeptide (**1**), the λmax of **1**, the amide absorption band, was shifted to a longer wavelength (Fig. 2). A solution containing solely the nonapeptide (**1**) or pentapeptides produced small and linear changes in $\Delta\lambda$max of UV spectra with increasing concentrations (*e.g.*, the dotted line for **7** in Fig. 3), indicating that the peptides do not form highly-ordered aggregation in an aqueous solution at the titration concentrations. As both the target sequence and the pentapeptides have a similar λmax, the titration data were corrected for the concentration-dependent shifts of the added peptides (Fig. 3). The constitution of the complex between **1** and **4** was determined by Job plot to be the 1:1 complex.[13] Thus, the titration data were obtained at the low concentrations of the pentapeptides where concentration-dependent shifts of λmax are negligible, and were analyzed as 1:1 complexes to produce K_d values (Table 1).[14] High affinities of all the selected pentapeptides (**4-9**) toward the target nonapeptide (**1**) have been demonstrated by *K*d values as high as 10^{-7} M in an aqueous buffer solution.

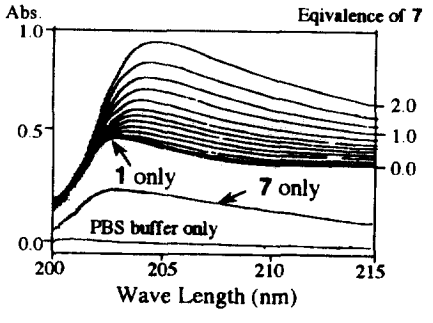

Fig. 2. UV Titration by the addition of **7**. The titration was performed using 6-7 μM of **1** in PBS buffer (pH 6.8) at 25 °C.

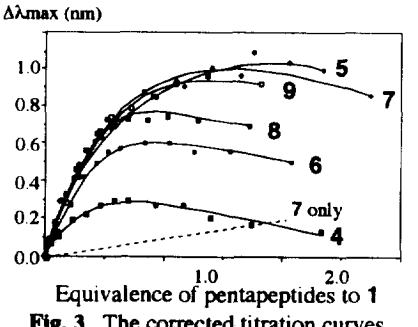

Fig. 3. The corrected titration curves. The titration data were corrected for the concentration-dependent shifts of the added peptides such as shown by dotted line for **7**.

This UV titration method might not give accurate K_d values, because small changes of λmax were monitored at the overlapping amide absorption region. However, it was also found that **1** showed changes in the ^{1}H-NMR in a PBS buffer at pH 6.8 containing 10% D_2O by the addition of **4** in a manner consistent with the formation of a complex, that is, four amide NH signals of **1** exhibited downfield shifts (Fig. 4).[15] Changes in both the NMR and UV spectra have consistently indicated that the amide groups were included in the complexation. NMR titration data were analyzed as 1:1 complexation to give K_d=5.6x10^{-5} M (Δδmax=0.142 ppm).[14] From observation of the precipitation at higher concentrations, it is apparent that the target peptide (**1**) assembles at the concentration for the NMR measurement. Such self-assembly of **1** should interfere with the complexation with the guest peptide, and then probably lead to an underestimation of the K_d value as compared to that by UV titration.

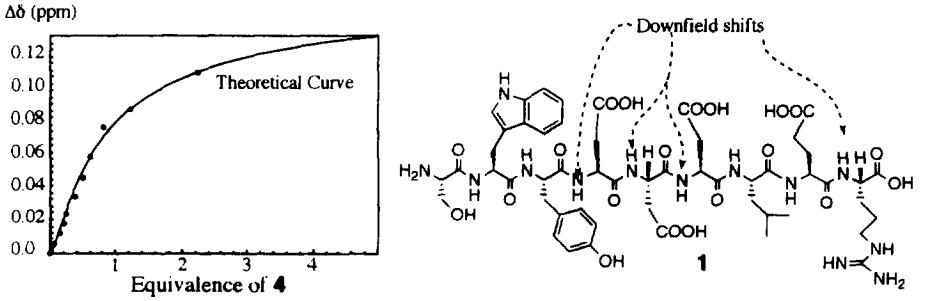

Fig. 4. ^{1}H-NMR Titration of **1** by the addition of **4**. The downfield shifts of one of the three aspartyl amide NH signals were plotted. The theoretical curve was drawn using *K*d and Δδmax values in Table 1.

Table 1. Calculated *K*d and δλmax (or Δδmax) values for the complexation between the host (**1**, **12**) and the guest peptides (**4-11**).[7]

Entry	Guest Peptides	Host Peptides	*K*d (10^{-7} M)	δλmax (nm)
1	Glu-Leu-Phe-Lys-Ala (**4**)	**1**	6	0.6
2	Glu-Leu-Phe-Lys-Ala (**4**)	**1**	560	0.14 (Δδmax: ppm)
3	Glu-Leu-Phe-Lys-Ala (**4**)	**12**	80	1.8
4	Tyr-Met-Glu-Gln-His (**5**)	**1**	7	1.6
5	Asp-Arg-Glu-Asp-Ile (**6**)	**1**	3	1.0
6	Phe-Ser-Asn-Ile-Thr (**7**)	**1**	1	1.6
7	Phe-Ser-Asn-Ile-Thr (**7**)	**12**	1	1.3
8	Ala-Ser-Ile-Phe-Glu (**8**)	**1**	33	1.4
9	Ala-Val-Thr-Glu-Asp (**9**)	**1**	16	1.0
10	Glu-Leu-Phe-Ala (**10**)	**1**	no complexation	0
11	Gly-Glu-Leu-Phe-Lys-Ala (**11**)	**1**	25	2.4

All titrations were monitored by UV spectroscopy using 6-7 μM of **1** except for run 2, in which ^{1}H-NMR measurements were done using a 0.6 mM solution of **1** in PBS buffer (pH 6.8) containing 10% of D_2O at 30 °C . The sequence of **12** is H-Ser-Trp-Tyr-Asp-Asp-Asp-Leu-Arg-OH.

Unfortunately, we could not determine the precise complex structures based on ^{1}H-NMR spectra, but some structural alteration of the guest peptides provided the following additional information. The

tetrapeptide (**10**) which lacks the lysyl residue of **4** did not cause any shifts of the λ_{max} of **1** (Table 1, entry 10), clearly showing that the lysyl residue plays a crucial role in producing the strong interaction between **1** and **4**. In contrast, recognition of the *N*-terminal of **4** was shown to be flexible from the fact that the additional glycyl residue to **4** at the *N*-terminal (**11**) altered the binding affinity slightly (entry 1 *vs* 11). The octapeptide (**12**) in which the glutamyl residue is lacking compared to **1** did not affect the binding with **7** but decreased the affinity toward **4** (entry 3 *vs* 7). It may be speculated from these results that ionic interaction between polar groups produce the strong interaction in combination with the hydrogen bondings of the amide NH groups, although interaction modes are dependent on both the host and the guest peptides.

In conclusion, we have identified recognition peptides for the target peptide (**1**) by combinatorial technologies using a solid-phase pentapeptide library and the magnetic beads covalently bound with the target sequence. Both detection and selection of the interacting beads could be performed easily by the use of magnetic beads. We will further apply the pentapeptides selected in this study to the recognition of the extracellular region of the dopamine D2 receptor.

References and Notes

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2) Some hypotheses to predict recognition molecules based on the target sequences. Bost, K. L.; Smith, E. M.; Blalock, J. E., *Proc. Natl. Acad. Sci. USA*, **1985**, *82*, 1372, Araga, S.; Le Boeuf, R. D.; Blalock, J. E., *ibid.*, **1993**, *90*, 8747, b) Ghiso, J.; Saball,E.; Frangione, B., *ibid.*, **1990**, *87*, 1288, c) de Gasparo, M.; Whitebread, S.; Einsle, K.; Heusser, C., *Biochem. J.*, **1989**, *261*, 310.

3) Peptide ligands for a small peptide-based receptor have been identified by combinatorial technologies, Boyce, R.; Li, G.; Nestler, H. P.; Suenaga, T. ; Still, C.; *J. Am. Chem. Soc.*, **1994**, *116*, 7955.

4) Lam, K. S.; Salmon, S. E.; Knapp, R. J., *Nature*, **1991**, *354*, 82. And a recent review on combinatorial technologies; Callop, M. A.; Barrett, R. W.; Dower W. J.; Fodor, S. P. A.; Gordon, E. M., *J. Med. Chem.*, **1994**, *37*, 1233.

5) Van Tol, H. H. M.; Bunzow, J. R.; Guan, H.-C.; Sunahara, R. K.; Seeman, P.; Niznik, H. B.; Civelli, O.; *Nature*, **1991**, *350*, 610. The D2 receptor consists of three domains; extracellular, transmembrane, and intracellular domains. The latter two domains interact with dopamine and G_S protein, respectively, but functions of the extracellular region have not been elucidated.

6) Chloromethylated polystyrene-divinyl benzene (2%, 100-200 mesh; Cl: 0.96 mmol/g) beads were used.

7) The single sequence constructed on one library bead and the magnetic beads were evidenced with an amino acid sequencer or by amino acid analysis, respectively.

8) Long Chain Alkyl Amino-MPG (500 Å, 5 μ), CPG INC (New Jersey, U.S.A.).

9) A recent example, Lonneborg, A., Sharma, P., Stougaard P., *PCR - Methods and Applications* , **1995**, *4*, S168.

10) One gram of library beads (*c.a.* 20^5 beads with different sequences) and 7.6 mg (0.64 μmol) of magnetic beads were used.

11) Ionic strength of the PBS solution was adjusted with NaCl to 0.14 M.

12) All the peptides used in this study were synthesized by the standard protocol of solid-phase synthesis using *N*-Fmoc amino acids, and were purified by reverse-phase HPLC (column: nacalai tesque 5C-18 MS, linear gradient; A:0.1% TFA H_2O, B; 0.1% TFA CH_3CN, flow rate: 1 ml/min, monitored at 220 nm). These peptides were quantitatively analyzed by ^1H NMR using crotonic acid as the internal standard. A PBS solution[9] contained 6-7 μM of the nonapeptide (**1**) was used for UV measurements at 25°C.

13) The total concentration was kept at 20 μM using **1** and **4**, and the complexation was monitored by UV spectrometer at 30°C.

14) The data were analyzed by the curve fitting method. Wilcox, C. S.; Cowart M. D., *Tetrahedron Lett.*, **1986**, *27*, 5563

15) ^1H-NMR measurements were done at 600 MHz at 30°C with presaturation of the water signal using a solution of 0.6 mM of **1**. The signals were assigned based on the gmqcosy and gtnroesy spectra.

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