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Quantitative screening of EGF receptor-binding peptides by using a peptide library with multiple fluorescent amino acids as fluorescent tags

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ABSTRACT

EGF receptor-binding peptides could be found by a peptide screening method using fifteen fluorescent amino acids as fluorescent tags. Of 225 peptides, we found an 8-mer peptide containing a dipeptide unit, Y–F, which was the strongest binding peptide to the EGF receptor.

Peptide screening methods such as the phage display method[1] and one-bead-one-compound (OBOC) method[2,3] have been widely used for identifying the peptides binding to target proteins. However, these methods essentially need very large carriers such as phages and beads to fix the peptides, and use of the carriers often has the drawback of peptides binding to target proteins not being able to be accurately found due to nonspecific interaction between target proteins and the carriers.[4]

To resolve this drawback, we have recently developed a new peptide screening method using multiple fluorescent amino acids as fluorescent tags instead of using such carriers.[5] By this method, target protein-binding peptides can be quantified from fluorescence intensity of each peptide labeled with a fluorescent amino acid, and accurate peptide screening is expected because target protein-binding peptides can be found by this method without the use of large carriers.

In this study, we carried out quantitative screening of peptides binding to the EGF receptor[6,7] (epidermal growth factor receptor = EGFR) by using this method. EGFR is a cell-surface receptor. Mutations affecting EGFR expression or activity can result in cancer. Therefore, an EGFR-binding peptide is an attractive medical tool, and some research groups have searched for such a peptide by using phage display methods.[8–11]

First, we prepared fifteen peptides containing a fluorescent amino acids[12] (fluorescent peptides) as one set of fluorescent peptide by conventional Fmoc-based solid-phase peptide synthesis.[13–16] The chemical structures of the peptides and the fluorescent amino acids[17] are shown in Figure 1. The sequences are Ac-EE-**Fl**-EE-Sp6-XXX-**Y1**-$\mathbf{Y_2}$-XXX-NH_2.[18] **Fl** indicates a fluorescent amino acid. Fifteen fluorescent amino acids are used in this study. To improve water solubility, four D-glutamic acids are connected with the fluorescent amino acid. X indicates an equimolar mixture of fifteen D-configurated natural amino acids other than Cys, Glu, Gly, Leu and Gln.[19] $\mathbf{Y_1}$ and $\mathbf{Y_2}$ are any one of the fifteen D-configurated natural amino acids.[20] The 8-mer peptide consisting of X, $\mathbf{Y_1}$ and $\mathbf{Y_2}$ is a library moiety. Sp6 (a long spacer consisting of ethylene glycol units) connects the fluorescent amino acid with the library moiety. Ac indicates an acetyl group. The C-terminals of the peptides are primary amides. We prepared in total 15 sets of fifteen fluorescent peptides (225 fluorescent peptides consisting of different $\mathbf{Y_1}$–$\mathbf{Y_2}$; Fig. S4, Supplementary data). We also prepared fifteen fluorescent peptides consisting of a random library moiety (Ac-EE-**Fl**-EE-Sp6-XXX-X-X-XXX-NH_2), and two-dimensional fluorescence (2D-FL) spectra of the fluorescent peptides were measured as component spectra as described below (Fig. S5, Supplementary data).

Next, to examine whether these fluorescent peptides can be discriminated and quantified by least-squares analysis from a 2D-FL spectrum of the mixture of fifteen fluorescent peptides on the basis of component spectra, we measured the 2D-FL spectrum of the mixture of fifteen fluorescent peptides prepared with various predetermined concentrations. And we estimated each concentration of the fluorescent peptides by aforementioned method (Fig. 2).[21] The calculated concentrations of fluorescent peptides were in fair agreement with the predetermined concentrations. It

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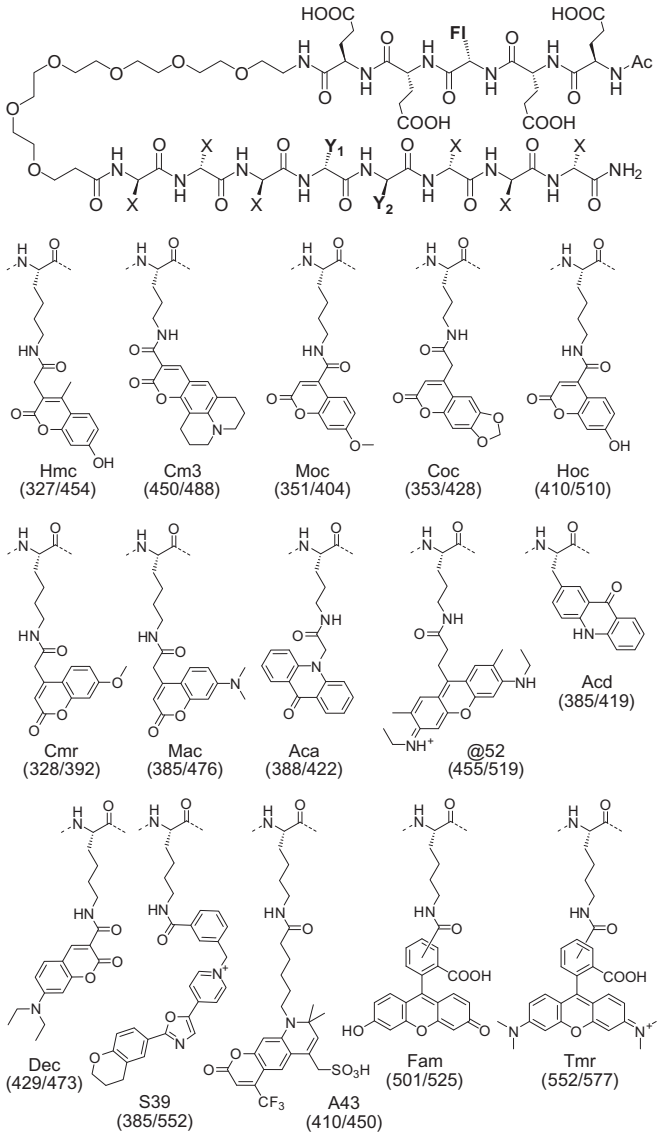

Figure 1. Chemical structure of the peptide modified with a fluorescent amino acid and chemical structures of fifteen fluorescent amino acids. Values in a parenthesis indicate maximum excitation/emission wavelengths obtained from the 2D-FL spectrum of each fluorescent amino acid in 70 mM HEPES buffer (pH 7.4)/methanol (1:1 (v/v)) at room temperature.

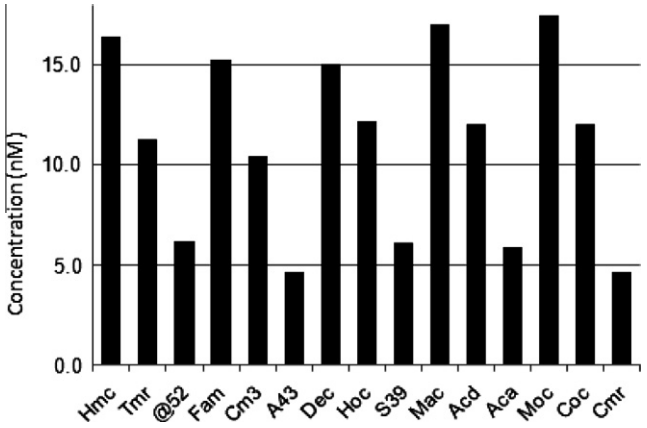

Figure 2. Concentrations of fifteen fluorescent peptides consisting of a random library moiety estimated from the 2D-FL spectrum of the mixture of peptides by least-squares analysis. Each concentration of **Hmc**, **Fam**, **Dec**, **Mac** and **Moc** is 15 nM. Each concentration of **Tmr**, **Cm3**, **Hoc**, **Acd** and **Coc** is 10 nM. Each concentration of **@52**, **A43**, **S39**, **Aca** and **Cmr** is 5 nM. The spectrum of the mixture and component spectra were measured in 70 mM HEPES-NaOH buffer (pH 7.4, 150 mM NaCl)/methanol (1:1 (v/v)).

pmol		Y_1														
		F	Y	W	I	V	M	A	T	S	N	K	H	R	D	P
Y_2	F	24	41	12	10	2	4	6	11	10	11	4	5	0	2	0
	Y	9	30	5	10	5	3	7	23	4	1	4	3	0	2	-1
	W	13	11	13	7	3	3	6	5	4	0	0	3	1	1	-1
	I	9	25	35	13	16	4	13	5	7	1	8	8	3	8	0
	V	13	14	12	-2	13	5	9	4	4	-5	6	4	2	2	0
	M	12	30	-3	3	4	8	17	8	4	1	2	9	0	-2	0
	A	8	4	2	3	5	1	6	5	-2	0	2	1	1	1	0
	T	6	5	5	5	3	1	2	4	1	1	6	1	0	2	0
	S	3	9	3	0	3	1	4	2	4	0	12	5	0	0	0
	N	3	-7	1	5	-2	0	2	0	0	-2	0	1	0	0	1
	K	4	-6	0	0	-8	1	1	1	-1	0	0	0	-1	0	0
	H	3	6	-17	2	4	1	8	4	-4	-5	2	-1	0	0	0
	R	3	-8	-13	-7	1	1	0	0	1	0	0	-2	-1	-1	0
	D	7	1	6	3	3	-2	3	-9	-1	0	0	1	0	-1	0
	P	1	3	27	1	2	0	0	0	0	0	0	-5	0	0	1

Figure 3. Map of binding amounts of the peptides to the EGFR estimated from 2D-FL spectra by least-squares analysis. Each value in cells indicates the amount of peptide binding to EGFR (pmol).

was shown that fifteen types of fluorescent peptides could be discriminated and quantified at the nanomolar order (pmol order).

Finally, peptides binding to the EGFR were selected and quantified by the method using these fluorescent peptides. In this study, the water-soluble ErbB1 (sErbB1; the extracellular domain of human EGFR) was used as an EGFR.[22] Fifteen fluorescent peptides were incubated with the EGFR in 70 mM HEPES-NaOH buffer (pH 7.4, 150 mM NaCl) at 4 °C for 2 h. Each final concentration of the peptides was 190 μM (4.5 nmol) and the concentration of EGFR was 8.6 μM (0.36 nmol). The fluorescent peptides binding to EGFR were recovered by gel filtration chromatography (Superdex™ 75 prep grade was purchased from GE Healthcare (London, UK).). Then the amounts of fluorescent peptides binding to the EGFR were determined with 2D-FL spectroscopy in 70 mM HEPES-NaOH buffer (pH 7.4, 150 mM NaCl)/methanol (1:1 (v/v)). The binding of each fluorescent peptide to EGFR was quantified by least-squares analysis from the 2D-FL spectrum. We repeated the aforementioned protocols for 15 sets of fifteen fluorescent peptides. These results are shown in Figure 3.

These fluorescent peptides were successfully estimated by this method, and it was shown that the amount of the peptide binding to the EGFR depends on a dipeptide unit, $\mathbf{Y_1}$–$\mathbf{Y_2}$, in the 8-mer peptide library. Many fluorescent peptides did not bind to the EGFR, but binding of some of the peptide to the EGFR could be detected.[23] Of the fluorescent peptides, Ac-EE-**Fam**-EE-Sp6-XXX-Y-F-XXX-NH_2 was found to show the strongest binding to the EGFR. The binding amount of the peptide was 41 pmol. The reverse sequence, F–Y, showed weak binding to the EGFR (9 pmol). It was clarified that the sequence is important for binding to the EGFR. Dipeptide units containing amino acids carrying an aromatic ring (F, Y and W) and a hydrophobic group (I and V) seemed to bind to the EGFR strongly. This result indicates that the peptides binding to EGFR were successfully discriminated and quantified by this method.

In summary, we synthesized 225 peptides modified with a fluorescent amino acid as fluorescent tags for screening EGFR-binding

peptides. Binding amounts of peptides to the EGFR were successfully quantified by this method. The 8-mer peptide containing a dipeptide unit, Y–F, was the strongest binding peptide to the EGFR. Determination of an 8-mer peptide sequence that binds to the EGFR by this method is currently underway.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.08.078.

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16. Three fluorescent amino acids (**Hmc**, **Dec** and **Coc**) were newly synthesized (see Supplementary data).
17. We selected positions of $\mathbf{Y_1}$ and $\mathbf{Y_2}$ in the center of the segment of library moiety because we assumed that molecular fluctuation at their positions is less (the amino acids strongly interact with the target).
18. Fifteen natural amino acids were chosen to assign fifteen fluorescent amino acids. Of twenty natural amino acids, Cys was removed to avoid intracyclization, dimerization or polymerization of peptides. Glu, Gly, Leu and Gln were removed to have amino acids of a similar property (Asp, Ala, Ile and Asn, respectively).
19. We selected D-configurated amino acids as the units composing fluorescent peptides because we assumed that resistance to proteolytic degradation of D-configurated peptides is superior to that of L-configurated peptides. We will utilize EGFR-binding peptides found by this method as medical tools in the future.
20. A 100-μL mixture of fluorescent peptides (concentration of each peptide being 5–15 nM) was measured by high-sensitive 2D-FL spectroscopy with a CCD camera (Photon design, Japan).
21. sErbB1 was obtained from sErbB1/293H cells provided by Professor Masaharu Seno, Okayama University.
22. Negative values may be experimental errors ascribed to 2D-FL spectrometer. The errors may be caused by deviations between component spectra and each spectrum of fluorescent peptides obtained by decomposing the objective spectrum.
23. Abbreviations used are as follows: Fmoc, 9-fluorenylmethoxycarbonyl; **Hmc**, *N*-ε-(7-hydroxy-4-methyl-coumarin-3-acetyl)-L-lysine; **Cm3**, *N*-δ-(coumarin 343-3-carbonyl)-L-ornithine; **Moc**, *N*-ε-(7-methoxycoumarin-4-carbonyl)-L-lysine; **Coc**, *N*-δ-(6-oxo-6*H*-[1,3]dioxolo[4,5-*g*]chromen-8-acetyl)-L-ornithine; **Hoc**, *N*-ε-(7-hydroxycoumarine-4-carbonyl)-L-lysine; **Cmr**, *N*-ε-(7-methoxycoumarin-4-acetyl)-L-lysine; **Mac**, *N*-ε-(7-dimethylaminocoumarin-4-acetyl)-L-lysine; **Acm**, *N*-δ-(9-oxoacridin-10(9*H*)-acetyl)-L-ornithine; **@52**, *N*-δ-{(*Z*)-*N*-(6-(ethylamino)-2,7,dimethyl-3*H*-xanthen-3-ylidene-9-ethylcarbonyl)}-L-ornithine; **Acd**, β-{2-[9(10*H*)-acridonyl]}-L-alanine; **Dec**, *N*-ε-(7-diethylaminocoumarin-3-carbonyl)-L-lysine; **S39**, *N*-ε-(4-(2-(chroman-6-yl) oxazol-5-yl)-1-benzylpyridinium-3-carbonyl)-L-lysine; **A43**, *N*-ε-{(8,8-dimethyl-2-oxo-4-(trifluoromethyl)-8,9-dihydro-2*H*-pyrano[3,2-*g*]quinolin-6-yl) methanesulfonic acid-9-pentylcarbonyl}-L-lysine; **Fam**, (fluorescein-5(6)-carbonyl)-L-lysine; **Tmr**, (tetramethylrhodamine-5(6)-carbonyl)-L-lysine.