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Angiotensin II inhibitory peptide found in the receptor sequence using peptide array

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Abstract

Peptide array consisting of hundreds of peptides spatially addressed and synthesized on a cellulose membrane support was used to screen ligand-inhibitory peptides. As a model, angiotensin II (Ang II), a significant peptide related to the treatment of cardiovascular diseases, was chosen as the target ligand. Peptide arrays covering the Ang II receptor type 1 sequence were prepared, and peptide domains with high affinity to the Ang II fluorescein conjugate were investigated. The peptide (VVIVIY) within the first transmembrane region exhibited the highest affinity to Ang II. The synthesized soluble VVIVIY peptide had an 84% inhibitory effect on Ang II-induced aorta contraction. These results indicate that our screening strategy utilizing peptide array is an effective approach for the peptide drug development.

Keywords: Peptide array; Angiotensin II; Peptide; Cellulose membrane support; AT1 receptor; Transmembrane; Antihypertension; High-throughput screening; Peptide drug

Recent developments in the identification of novel drug targets from benefits of genome projects have created an increasing demand for synthetic peptides and peptide mimetic compounds. In such background, The SPOT-synthesis methodology developed by Frank [1] has achieved efficient and high-throughput screening of peptides for biomedical and pharmaceutical research. Based on the SPOT-technique, peptide arrays, which are coherent membrane-supported peptide libraries, have become powerful tools in studies of molecular recognition events and identification of biologically active peptides [2–4].

The basic methodology of constructing a peptide array involves spotting of small volumes of activated amino acid solution to addressed positions on membrane supports, and elongation of peptide sequences by Fmoc chemistry known as the solid-phase peptide synthesis technique. Peptide arrays have been applied to a wide range of high-throughput peptide studies. Antibody–antigen epitope mapping was the first and is the most frequent application. Other applications have been extended to peptide–metal ion binding [5], peptide–nucleic acid binding [6], protease cleavage studies [7], and kinase phosphorylation studies [8,9]. However, peptide arrays have not commonly been used to obtain peptide–peptide interactive data for peptide design or development of peptide medicines.

In the present study, we have applied to the high-throughput peptide assay system to accelerate the screening of bioactive inhibitory peptides as candidate peptide drugs. As a model case to test our inhibitory-peptide screening strategy, we chose angiotensin II (Ang II), which is significantly involved in cardiovascular diseases such as hypertension, as the model target.

Ang II, a short 8-mer peptide with the sequence Asp-Arg-Val-Tyr-Val-His-Pro-Phe, is one of the strongest vasopressor substances that regulate the cardiovascular system and blood pressure. Ang II causes vasoconstriction and salt and water reabsorption, thus affecting

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electrolyte balance, blood volume, and arterial blood pressure. As a potent vasoconstrictor hormone, Ang II has been implicated to play a critical role in hypertension, congestive heart failure, and a number of other related clinical conditions [10].

Ang II initiates a specific signal transduction pathway by binding to specific high-affinity receptors present on the cell membrane. The high-affinity receptors for Ang II belong to the seven-transmembrane G protein coupled receptor superfamily and are defined in at least two distinct receptor subtypes: type 1 (AT1) and type 2 (AT2) [11]. These receptors have been identified in a number of peripheral tissues, including those of the aorta, heart, mesenteric artery, adrenal cortex, liver, uterus, bladder, and pituitary. Results of site-directed mutagenesis studies associated with biochemical and pharmacological experiments have suggested that more than 40 residues in AT1 are sensitive either to ligand binding or to signal transduction [12–14]. 3D models of AT1 and its complexes with ligand have been developed on the bases of site-directed mutagenesis experiments [14]. However, due to the complexity of their seven-transmembrane structure, details of the ligand-associating domains of these receptors remain unclear.

In hypertension therapy, chemical substances such as losartan (which directly target Ang II receptors) have proven to be effective, with less side effects than angiotensin-converting enzyme (ACE) inhibitors (which inhibit the enzymatic activity that forms Ang II). However, by targeting receptors that also exist in other peripheral tissues, prolonged clinical treatment with receptor antagonist could lead to raise plasma Ang II levels and may act on other receptor subtypes [10]. Thus, antagonists that target the ligand itself rather than the receptor may act as more selective drugs that do not affect other supportive functions of the receptor, and may result in milder effects to the patient.

The aim of the present study was to establish an effective method of screening ligand-inhibitory peptides, as candidates of antihypertensive peptide drugs, from the peptide array technique utilizing receptor sequence. For the first time, we report a peptide domain, in the first transmembrane region of AT1, that exhibits high affinity to Ang II, and the inhibitory effect of the soluble peptide on Ang II-induced vascular contraction. Our strategy provides interactive data that can clarify the complex mechanism of ligand–receptor interactions and facilitate high-throughput screening and design of antagonists as candidate peptide drugs.

Materials and methods

Preparation of synthetic peptide array. The cellulose membrane (grade 542, Whatman, Maidstone, UK) was activated by β-alanine as spacer amino groups. Standard Fmoc-chemistry was used in accordance with the instructions of the manufacturer (Intavis), with some modifications. The activated amino acids were spotted using a peptide auto-spotter (ASP222, IntavisAG, Koeln, Germany). Activated amino acids, at a concentration of 0.25 M, were spotted with an interval of 2.0 mm. The membrane was washed with *N*,*N*′-dimethylformamide (DMF) and deprotected with 20% piperidine. Additional washes were performed with DMF, followed by washing with methanol. These steps were performed in this order with every elongation step. Final removal of side-chain protecting groups was performed by washing the membrane with the reagent (*m*-cresol:thioanisole:ethanedithiol:trifluoroacetic acid (TFA) = 1:6:3:40) for 4 h. After cleavage, the membrane was washed several times with diethyl ether and methanol, and was allowed to dry.

Spot screening on the membrane. The peptide array was washed several times with phosphate-buffered saline (PBS; pH 7.0). The washed array was soaked in 1% bovine serum albumin in PBS for 12 h for blocking. Ang II–fluorescein conjugate (Molecular Probe, Leiden, Netherlands) was used as the probe, according to the previous study reported by Hein et al. [15]. After blocking, the array was incubated for 1 h in PBS containing Ang II–fluorescein conjugate at a final concentration of 1 μg/ml, with slow rotation. Fluorescence of the peptide spots was detected using FLA-3000 (Fujifilm, Tokyo, Japan) with a 494 nm/522 nm filter. The scanned image was analyzed with Genepix 3.0 (Axon Instruments, CA, USA) and fluorescent intensity of each spot was calibrated. Each array was designed to contain duplicate or triplet spots, and the average of fluorescent intensities from three to five different membranes was calculated. The relative fluorescent intensity was calculated as the ratio of measured fluorescent intensity to the control peptide (AGAGAG) fluorescent intensity.

Soluble peptide synthesis and purification. To obtain soluble peptides, solid-phase peptide synthesis was performed using Fmoc-PAL-PEG-PS high-load resin (Perseptive Biosystems, Warrington, UK) as the support. Side chain deprotection of the peptide was performed with the same deprotection process described in the former section. In the deprotection step, synthesized peptides were released from the resin. After precipitation of peptides with diethyl ether, the crude pellet was dissolved in buffer A (water containing 0.1% TFA) and subjected to reverse-phase chromatography using a C-18 column. Elution was performed with buffer B (acetonitrile containing 0.1% TFA) and the major fractions containing soluble peptide were collected. The collected fractions were analyzed using Voyager-DE PRO (Applied Biosystems, Foster city, CA, USA) and the fraction with mass spectrometry corresponding to the inhibitory peptide's molecular weight was lyophilized.

Vascular contractile assay. Male rats (250–300 g) were anesthetized with pentobarbital sodium (Nembutal, 40 mg/kg, i.v.) and sacrificed after rapid exsanguination. The thoracic aorta was isolated and immersed in cold Krebs–Hensleit bicarbonate (KHB) solution of the following composition: NaCl, 114 mM; KCl, 4.7 mM; $CaCl_2$, 2.5 mM; $MgCl_2$, 1.2 mM; KH_2PO_4, 1.2 mM; $NaHCO_3$, 25 mM; and dextrose, 10 mM. The aorta was cut into rings (diameter, 1.7–2 mm; length, 1.5–2.0 mm) and the endothelium of the preparations was removed by gently rubbing the intimal surface with a metal wire. Each aorta ring was stretched to resting tension of 1.6 g in 4 ml of warmed (37 °C) KHB solution containing 3 μM propranolol to avoid β-adrenoceptor-mediated responses and oxygenated with a 95% O_2 and 5% CO_2 gas mixture for isometric tension recording. After an equilibration period of 60 min, conditioning contractions were induced 3 times with 10 μl norepinephrine for 5–10 min at 40 min intervals. Ang II was dissolved in PBS and the inhibitory peptide candidate was dissolved in dimethyl sulfoxide (DMSO). The equivalent volume of Ang II solution and DMSO with or without the inhibitory peptide (100 equivalent molar ratio) was preincubated for 12 h at 25 °C. The mixture was injected into the magnus holding stretched rat aorta ring, for a final Ang II concentration of 140 nM. Isometric tension was recorded as a function of time on a strip chart recorder (Nihon Kohden Kogyo, Tokyo, Japan). The time course of force development during Ang II-induced

contractions in the presence of inhibitory peptide was expressed as relative contractile response (response to sample/response to norepinephrine).

Results

Screening of peptide domains with high affinity to angiotensin II

To begin screening of Ang II-binding peptide sequences, we hypothesized that the peptide domain with strong affinity to the target ligand, Ang II, would exist in the sequence of high-affinity receptor with high probability. In accordance with this hypothesis, we designed a peptide array covering the entire AT1 receptor sequence (Fig. 1). As shown in the scheme (Fig. 1), a sequence of 359 residues from the AT1 sequence (Swiss-Prot Accession No. P30556) was sectioned into 8-mer peptide fragments with 1 residue shift from the N-terminal, resulting in 352 spots, designated as ATR1 array.

Hybridization of Ang II–fluorescein conjugate was performed using ATR1 array. As shown in Fig. 2, several spots with strong fluorescent intensities were observed. The four continuous spots detected at row C and columns 2–5 showed especially high signal intensities. These continuously positive spots indicate the existence of significant overlapping domain related to ligand

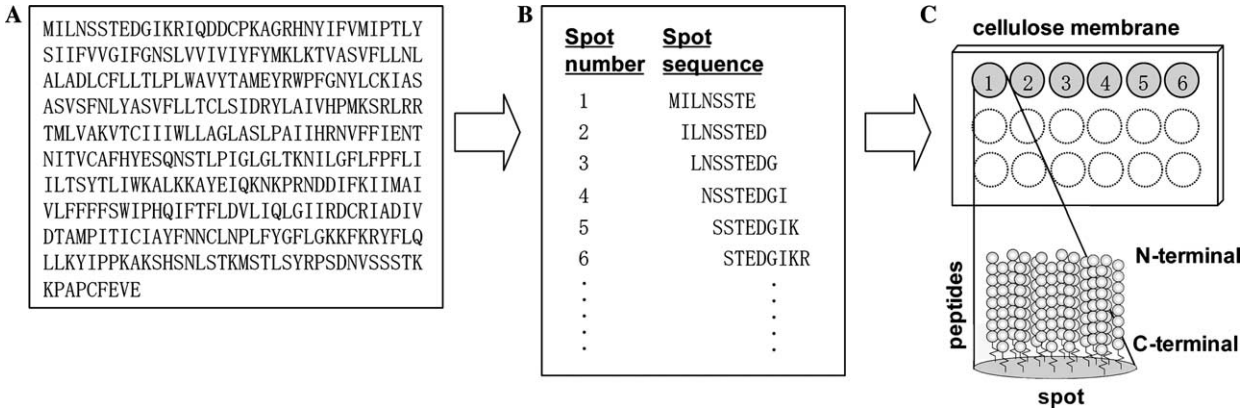


Fig. 1. Scheme for designing peptide array (ATR1 array) covering angiotensin II receptor type 1 sequence. (A) Sequence of angiotensin II receptor type 1. (B) An example of peptide array designed from the receptor sequence (8-mer/1 amino acid shift). (C) The spotting scheme on the cellulose membrane support and the spot peptide image.

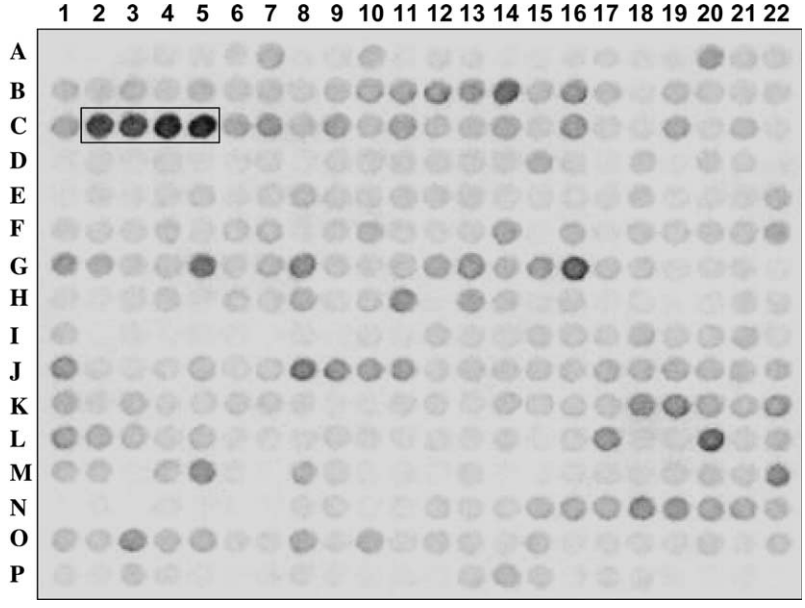


Fig. 2. Peptide spots on angiotensin II receptor type 1 array (ATR1 array) binding to angiotensin II–fluorescein conjugate probe. Peptides were synthesized with a length of 8-mer. Letters on the left-hand side of the array indicate rows. Numbers on the upper side of the array indicate columns. Squares indicate continuous spots showing high fluorescent intensity.

Table 1
Profile of 15 spot peptides with the highest fluorescent ratio on angiotensin II receptor type 1 array

Row	Column	Peptide sequence	Relative fluorescent ratio	Hydrophobicity	van der Waals volume	Electrical effect
C	4	LVVIVIYF	18.71 (2.83)	26.8	33.4	0.09
C	5	VVIVIYFY	17.97 (4.85)	21.7	35.8	0.13
C	2	NSLVVIVI	14.53 (3.17)	21.1	25.6	0.17
C	3	SLVVIVIY	14.48 (2.97)	23.3	29.1	0.14
G	16	TCIIIWLL	13.73 (1.71)	22.0	33.1	0.11
J	8	FPFLIILT	12.00 (0.11)	19.7	33.1	0.12
B	14	SIIFVVGI	11.91 (2.75)	23.4	25.5	0.16
L	20	LDVLIQLG	11.29 (2.60)	12.7	25.7	0.17
G	5	RLRRTMLV	10.47 (2.22)	−0.5	36.4	0.19
M	5	GIIRDCRI	9.31 (0.11)	3.1	29.5	0.32
G	8	RTMLVAKV	9.06 (1.47)	6.8	28.9	0.12
K	19	FKIIMAIV	8.82 (0.48)	20.2	31.1	0.07
J	9	PFLIILTS	8.76 (0.04)	16.2	28.8	0.17
M	22	ITICIAYF	8.39 (1.90)	18.5	30.4	0.21
H	11	IHRNVFFI	8.33 (0.94)	7.4	36.5	0.29

Values in brackets are the SD of relative fluorescent ratios ($N = 6$).

binding. The four continuous spots all contained the sequence VVIVI. Identical results were obtained with high reproducibility using three different arrays containing duplicate spots.

Table 1 shows the profile list of the 15 spot sequences with the highest signal intensity. Fluorescent intensity was calculated as the average of three different arrays containing duplicate spotted areas (= 6 spots), and the average fluorescent intensity was compared with the intensity of the control peptide (AGAGAG) to yield the relative fluorescent ratio. The underlined sequences in Table 1 are the positions positively identified on the ATR1 array as continuous spots (Fig. 2). Other sequences with higher relative fluorescent ratio are listed together with their peptide physical properties, including total hydrophobicity [16], van der Waals volume [17], and electrical effect [17]. We found no correlation between relative fluorescent ratio and peptide properties significantly involved in peptide interactions. This indicates that interaction between Ang II and peptides on the peptide array is not due to non-specific physical properties, but is specific to the sequence.

Specificity of the angiotensin II-binding peptide identified by peptide array

The effect of peptide length on binding affinity to Ang II was examined. We designed a peptide array (DifL array) with peptides of different lengths (3–8 mer) covering the sequence of VGIFGNSLVVIVIYFYMKLK, which includes the continuously positive sequences obtained from the ATR1 array. As shown in Fig. 3, peptides longer than 6-mer exhibited continuously high fluorescent intensities similar to those observed in the ATR1 array results (Fig. 2). With the shorter peptides (3–5 mer), high affinity to the Ang II probe was not clearly observed. These results indicated that a minimum length of 6-mer was required for affinity to Ang II. Fig. 3A shows the detailed fluorescent signal intensity profile of the DifL array (6–8 mer). The overlapping sequence of the three spots with the highest affinity in each length was VVIVIY (Val-Val-Ile-Val-Ile-Tyr).

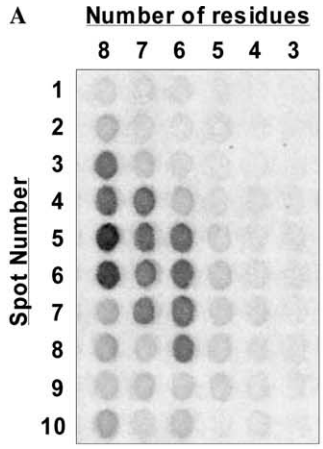
To confirm the sequence specificity of the interaction between Ang II and VVIVIY peptide, an inhibition assay was performed by preincubating the peptide array with non-labeled Ang II as a competitor to the probe. In Fig. 4A, the profile of the peptide array covering the sequence VGIFGNSLVVIVIYFYMKLKTVA, which includes the ligand-binding sequence VVIVIY, is described. Hybridization with the Ang II–fluorescein conjugate probe resulted in an apparent decrease in affinity to Ang II, in the presence of non-labeled Ang II. This result indicates that the VVIVIY peptide binds specifically to Ang II without interacting non-specifically with the fluorescein (Figs. 4B and C). On another array, we synthesized the peptide YIVIVV, which is the reverse of the VVIVIY sequence and has the same physical properties. We examined the affinity of YIVIVV to the Ang II–fluorescein conjugate probe. The YIVIVV peptide had diminished the affinity to the probe (data not shown), indicating that the affinity of the screened peptide VVIVIY to Ang II was sequentially specific.

Consequently, we regarded VVIVIY as a novel screened peptide domain that is a candidate Ang II-targeting antagonist.

Rat aorta contractile assay to confirm the biological effect of VVIVIY

To test the inhibitory effect of the candidate VVIVIY peptide on Ang II activity, soluble VVIVIY peptide was synthesized and rat aorta contractile assay was performed. The VVIVIY peptide was preincubated with Ang II for 12 h and then applied to a rat aorta ring kept

A



B

		8-mer			7-mer			6-mer		
		Peptide sequence	Relative fluorescent ratio	Hydro-phobicity	Peptide sequence	Relative fluorescent ratio	Hydro-phobicity	Peptide sequence	Relative fluorescent ratio	Hydro-phobicity
Spot number	1	FGNSLVVI	1.58 (0.09)	14.7	FGNSLVV	1.32 (0.19)	10.2	FGNSLV	1.40 (0.11)	6.0
	2	GNSLVVIV	3.72 (0.30)	16.2	GNSLVVI	1.30 (0.15)	12.0	GNSLVV	0.88 (0.22)	7.5
	3	NSLVVIVI	13.22 (1.00)	21.1	NSLVVIV	2.98 (0.25)	16.6	NSLVVI	1.11 (0.09)	12.4
	4	SLVVIVIY	14.46 (2.19)	23.3	SLVVIVI	12.11 (0.35)	24.6	SLVVIV	3.16 (0.36)	20.1
	5	LVVIVIYF	21.54 (2.60)	26.8	LVVIVIY	13.81 (2.87)	24.1	LVVIVI	12.42 (0.32)	25.4
	6	VVIVIYFY	20.24 (3.20)	21.7	VVIVIYF	11.56 (1.58)	23.0	VVIVIY	13.97 (1.12)	20.3
	7	VIVIYFYM	4.62 (0.43)	19.4	VIVIYFY	11.22 (0.66)	17.5	VIVIYF	11.79 (0.33)	18.8
	8	IVIYFYMK	5.81 (0.73)	11.3	IVIYFYM	2.58 (0.15)	15.2	IVIYFY	12.07 (0.05)	13.3
	9	VIYFYMKL	2.89 (0.38)	10.6	VIYFYMK	3.37 (0.08)	6.8	VIYFYM	2.70 (0.12)	10.7
	10	IYFYMKLK	6.50 (0.62)	2.5	IYFYMKL	1.87 (0.20)	6.4	IYFYMK	3.45 (0.06)	2.6

Fig. 3. Effects of peptide length and region on binding affinity to angiotensin II–fluorescein conjugate on peptide array (DifL array). (A) Hybridization result with angiotensin II–fluorescein conjugate probe. (B) Profile of DifL array results (6, 7, and 8-mer). Values in brackets are the SD of relative fluorescent ratios ($N = 10$). Hatched cell shows the peptide sequence with relative fluorescent ratio greater than 10.

A

Row	Column	Peptide sequence
A	1	VGIFGNSL
A	2	GIFGNSLV
A	3	IFGNSLVV
A	4	FGNSLVVI
A	5	GNSLVVIV
A	6	NSLVVIVI
A	7	SLVVIVIY
A	8	LVVIVIYF
B	1	VVIVIYFY
B	2	VIVIYFYM
B	3	IVIYFYMK
B	4	VIYFYMKL
B	5	IYFYMKLK
B	6	YFYMKLKT
B	7	FYMKLKTV
B	8	YMKLKTVA

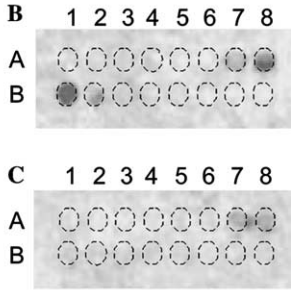


Fig. 4. Specificities of peptides binding to the angiotensin II–fluorescein conjugate probe on peptide array. (A) Peptide profile of the array used for the binding specificity assay. (B) Hybridization result with angiotensin II–fluorescein conjugate only. (C) Hybridization results with angiotensin II–fluorescein conjugate preincubated with unlabeled angiotensin II (100 μg/ml).

in tension. Ang II (final concentration, 140 nM) provoked a strong vascular contractile response when mixed with the inhibitory peptide dissolvent DMSO, however the contractile response was inhibited in the presence of VVIVIY (14 μM) (Table 2). This experiment was repeated in five independent rat aorta assays. The average

Table 2
Vascular contraction inhibitory effect of the soluble VVIVIY

	Angiotensin II final concentration = 140 (nM)
(Ang II + DMSO) response/ norepinephrine response	0.91 (0.3)
(Ang II + VVIVIY) response/ norepinephrine response	0.15 (0.2)
(VVIVIY) response/ norepinephrine response	0.0 (0.0)

The numbers in brackets are the SD of relative contraction responses ($N = 5$).

relative inhibition rate was 84% of the control response. The Ang II concentration was chosen to produce a high contractile response in the rat aorta ring. A mixture of VVIVIY peptide with the Ang II dissolvent PBS had no effect on the rat aorta (Table 2). These results strongly suggest that the VVIVIY peptide, obtained from our screening methodology using peptide array, has potential as a peptide drug for hypertension therapy.

Discussion

In the present study, for the first time, peptide-mapping using a synthetic peptide array was performed to find the peptide domain with high affinity to Ang II in the entire AT1 sequence. An octamer-peptide array covering the AT1 receptor sequence was designed (Fig. 1) and hybridized with Ang II–fluorescein conjugate as a probe. After 1 h of hybridization, several peptides showing high fluorescent intensity were obtained (Fig. 2). The peptide domain NSLVVIVIYFY, which was covered by the continuous spots and showed high fluorescent intensities, was located near the carboxyl-terminal end of the transmembrane (TM)-1 region.

First, some experiments with peptide agonists to AT1 receptor have suggested that the extracellular region of the receptor contributes to the ligand–receptor binding [18]. However, more recent studies have demonstrated that most other peptide hormone receptors contain a binding pocket formed by transmembrane helices [19–21]. These latter findings are consistent with the present results, in which a sequence in the transmembrane region showed high affinity to Ang II. Although it is known that a pocket in the receptor for Ang II binding is between TM-3, TM-5, TM-6, and TM-7, and there are no previous reports indicating that a sequence in the TM-1 region binds to Ang II. Since the X-ray crystallography data defining the conformational structure of the complex of AT1 and Ang II are still not obtained, the possibility that the TM-1 region plays a role in ligand–receptor binding attraction or stability cannot be ruled out.

Fig. 5 summarizes the positively binding peptide domains that showed strong fluorescent intensities in the present study. The solid lines over the amino acid sequence indicate the peptide domains that showed a relative fluorescent ratio greater than 6.0. The nine bald residues with residue numbers comprise the Ang II contact points, which have been confirmed by mutagenesis studies. The residues that have been found to be involved in Ang II binding in mutagenesis studies are Lys199 (TM-5) [22], Asp281 (TM-7) [15], His183 (extracellular loop 2) [21], Arg167 (extracellular loop 3) [23], Phe259 (TM-6) and Asp263 (extracellular loop 4) [20], Ile172 (extracellular loop 3) [24], Phe293 (TM-7), and Asn294 (TM-7) [25]. Lys102 (TM-3) and Ser105 (TM-3) [19,23] are also reportedly involved in binding of Ang II to the receptor, although it is unclear whether these residues directly interact with Ang II. When the present peptide array results were compared with results of the mutagenesis studies, we found that six residues were overlapping. This surprising finding suggests that some peptide domains interact with Ang II in a manner similar to their behavior in native receptor conformation. Thus, the present peptide array method provides interactive information that can facilitate understanding

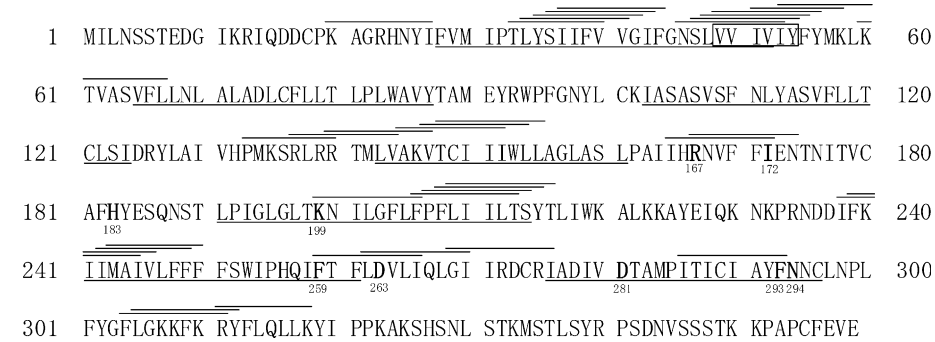


Fig. 5. Summary of the results of angiotensin II receptor type 1 peptide array and previously reported points of contact with the ligand. Underlines indicate the theoretical transmembrane domains. Solid lines over the sequence indicate peptides with a relative fluorescent ratio greater than 6.0. Bold letters with numbers indicate the residues that have been found to be involved in angiotensin II binding in previous mutagenesis studies. Solid squares indicate ligand-binding peptide domain (VVIVIY) obtained from the peptide array screening.

about complex protein interactions and may be able to identify functional domains common to several different seven-transmembrane receptors.

Compared with the other peptide screening method such as phage display, our strategy utilizing peptide array has three advantages for development of peptide drugs. First, the peptide array does not require the sequencing process involved in the standard phage display technique, and thus provides faster high-throughput screening. Second, the peptide array functions not only as a screening tool, but also functions as an effective tool for examining the effects of amino acid substitution and elongation on affinity. The effects of length starting from the seed sequence can be easily examined by peptide array (Fig. 3). Such flexibility of the peptide array is advantageous for peptide design processes such as chemical modification or amino acid substitution in non-natural amino acids. Third, the screening data obtained from the peptide array provide more information applicable to peptide design than does phage display screening. Because every peptide sequence is already addressed on the array, the assay provides binding data for all the peptides on the array, including both positive and negative results. With such informative data, the relationship between the binding affinity and peptide sequence data can be analyzed by various bioinformatic-modeling methods. Computational algorithms such as neural networks or hidden Markov models have shown high accuracy with such supervised data (containing positive results and negative results) [26]. These advantages of the peptide array strongly indicate that it is an effective screening method for the use in bioinformatic peptide drug studies.

The present results indicated the effectiveness of our peptide array strategy in the search for ligand-binding peptides. We used Ang II as the model target to obtain seed peptides with antihypertensive effect and found the candidate peptide VVIVIY from the sequence of AT1. Our strategy for screening candidate peptide drug seeds has high potential for the search for peptides that target other ligands such as hormones, cytokines, or other signaling peptides. Thus, we conclude that the present synthetic peptide array screening strategy is an effective high-throughput screening method for use in peptide drug development.

Acknowledgments

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