Structure–Antiviral Activity Relationships of Cecropin A-Magainin 2 Hybrid Peptide and its Analogues

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Abstract: In order to elucidate the structure–antiviral activity relationship of cecropin A (1–8)-magainin 2 (1–12) (termed CA-MA) hybrid peptide, several analogues with amino acid substitutions were synthesized. In a previous study, it was shown that serine at position 16 in CA-MA hybrid peptide was very important for antimicrobial activity. Analogues were designed to increase the hydrophobic property by substituting a hydrophobic amino acid residue (S → A, V, F or W, position 16) in the CA-MA hybrid peptide. In this study, the structure–antiviral activity relationships of CA-MA and its analogues were investigated. In particular, substitution of Ser with a hydrophobic amino acid, Val, Phe or Trp at position 16 caused a dramatic increase in the virus–cell fusion inhibitory activity. These results suggested that the hydrophobicity at position 16 in the hydrophobic region of CA-MA is important for potent antiviral activity. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antiviral activity; cecropin A (1–8)-magainin 2 (1–12); hydrophobicity

INTRODUCTION

It is generally recognized that antimicrobial peptides play an important role in the innate host defence mechanism of most living organisms including plants [1], insects [2–4], amphibians [5] and mammals [6]. These antimicrobial peptides possess

a potent antibiotic activity against bacteria, fungi and even certain enveloped viruses [6]. Most locations that are in contact with invading microbes are known to express antimicrobial peptides constitutively or on the challenge of several pathogens. Representative antimicrobial peptides are cecropin [7] and drosomycin [8] from insects, magainin [9] and melittin [10] from honeybees, α- and β-defensin from human neutrophils [11] and plant defensin [1] from plants. Despite the great diversity in the primary structures of these peptides, most antibiotic peptides are cationic at physiological pH and show the amphipathic characteristic of adopting α-helix and β-sheet structures in hydrophobic environments [12–13]. Among these, cecropin A (CA) identified from the cecropia moth is very active against Gram-negative bacteria, but less active

against Gram-positive bacteria [14]. Magainin 2 (MA) isolated from frog skin acts on both Gram-positive and Gram-negative bacteria, fungi and protozoa [9]. The hybrid peptides, which are composed of the *N*-terminal region of CA and MA, have been shown to have improved antimicrobial activity when compared with their parental peptides [15–16]. The CA-MA was used as the template peptide in this study. From the α-helical wheel diagram of the CA-MA hybrid peptide (Figure 1), several analogues were designed and synthesized by the solid phase method. In the present study, the relationships between the peptide structure and antiviral activity were investigated.

MATERIALS AND METHODS

Peptide Synthesis and Purification

The peptides were synthesized by the solid phase method using Fmoc(9-fluorenyl-methoxycarbonyl)-chemistry [17]. Rink amide 4-methyl benzhydrylamine (MBHA) resin (0.55 mmol/g) was used as the support to obtain a *C*-terminal amidate peptide. The coupling of Fmoc-amino acids was performed with N-hydroxybenzotriazole (HOBt) and dicyclohexylcarbodiimide (DCC). Amino acid side chains were protected as follows: *tert*-butyl (Asp), trityl (Gln), *tert*-butyloxycarbonyl (Lys). Deprotection and

Figure 1   The α-helical wheel diagrams of CA(1–8)-MA(1–12) hybrid peptide. The arrow indicates the amino acid residues substituted in their analogue peptides.

cleavage from the resin were carried out using a mixture of trifluoroacetic acid, phenol, water, thioanisole, 1, 2-ethanedithiol and triisopropylsilane (88 : 2.5 : 2.5 : 2.5 : 2.5 : 2.0, v/v) for 2 h at room temperature. The crude peptide was then repeatedly washed with diethylether, dried *in vacuo* and purified by a reversed-phase preparative HPLC on a Waters 15 μm Deltapak C_{18} column (19 × 30 cm). The purity of the peptide was checked by analytical reversed-phase HPLC on an Ultrasphere C_{18} column (Beckman, USA), 4.6 × 25 cm. The purified peptides were hydrolysed with 6 N HCl at 110 °C for 22 h, and then dried *in vacuo*. The residues were dissolved in 0.02 N HCl and subjected to amino acid analysis (Hitachi Model, 8500 A, Japan). The peptide concentration was determined by amino acid analysis. The molecular weights of the synthetic peptide were determined by MALDI-MASS.

Cells and Viruses

CD4 positive HeLa cells carrying a CD4 protein on its surface were cultured with Ham's F12 medium supplemented with 10% (v/v) heat-inactivated newborn calf serum (NBCS), 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate and $NaHCO_3$ (1.176 g/l). Syncytia formation activity of the $CD4^+$ HeLa cells with vPE 16 was constant throughout the experiments. The cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2. The cells were subcultured twice a week at 5×10^5 cells/ml. Recombinant vPE 16 was prepared from a vaccinia virus which expresses HIV-1 envelope protein gp 120 and gp 41 on its surface. The stock of vPE 16 was prepared from culture supernatant of vPE 16 infected Vero cells. The virus titre of the supernatant was determined using a plaque assay. The virus stock was stored as aliquots at −80 °C until used. Both the $CD4^+$ HeLa cells and recombinant vPE 16 were kindly provided by Dr M. Nishijima at NIH in Japan.

Syncytia Formation Inhibition Assay

The assay using $CD4^+$ HeLa cells and vPE 16 was carried out as described by Tochikura *et al* [18] with some modifications [19–20]. $CD4^+$ HeLa cells (2×10^5 cells/ml) in log-phase were seeded in 12-well culture plates (3 ml cell suspension/well). Three days later, the plates were washed with fresh medium to remove unadhered cells and then 0.2 ml of culture medium including test peptides was added. The culture plates were incubated at
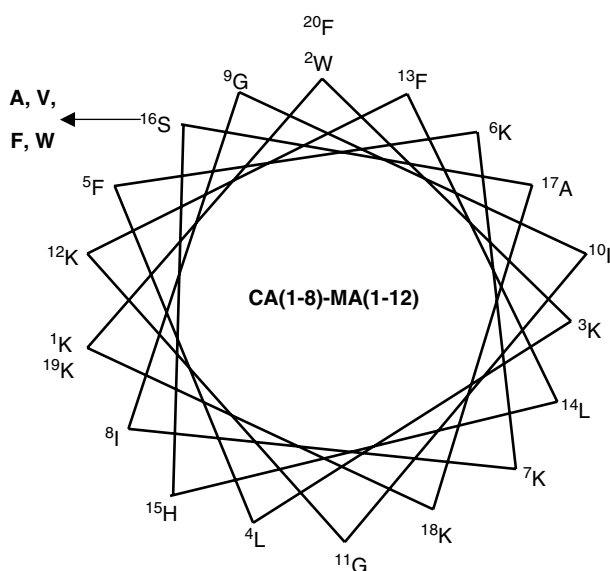
37 °C in a humidified atmosphere with 5% CO_2 for 30 min. Then 10 µl of diluted vPE 16 (7.3×10^2 PFU) was added and incubated for an additional 30 min. After incubation, 0.8 ml of culture medium was added and again incubated. After 16–20 h of incubation, syncytia formation was observed under the microscope. The fusion index (FI) and the percentage of fusion inhibition were calculated as follows

$$FI = \frac{\text{Total number of nuclei}}{\text{Total number of cells}} - 1$$

$$\% \text{ fusion inhibition} = \left(1 - \frac{FI_1}{FI_2}\right) \times 100$$

where FI_1 is the fusion index of the test sample and FI_2 is the fusion index of the control sample.

Circular Dichroism Analysis

A CD spectra of the peptide was recorded using a Jasco J720 spectropolarimeter (Japan). The sample was maintained at 25 °C during analysis. Four scans per sample were performed over a wavelength range 190–250 nm at 0.1 nm intervals. The spectra were measured in 10 mM sodium phosphate buffer, pH 7.0, 50% (v/v) TFE in 10 mM sodium phosphate buffer, pH 7.0 and 30 mM SDS in 10 mM sodium phosphate buffer, pH 7.0, respectively, using a 1 mm pathlength cell. The peptide concentrations were 100 µg/ml. The mean residue ellipticity, $[\theta]$, is given in deg cm^2/dmol: $[\theta] = [\theta]_{obs}$ (MRW/10lc), where $[\theta]_{obs}$ is the ellipticity measured in millidegrees, MRW is the mean residue molecular weight of the peptide, c is the concentration of the sample in mg/ml, and l is the optical pathlength of the cell in cm. The percentage helicity of the peptide was calculated with the following equation [21]

$$\% \text{ helicity} = 100([\theta]_{222} - [\theta]_2^0)/[\theta]_{222}^{100}$$

where θ is the experimentally observed mean residue ellipticity at 222 nm. Values for $[\theta]_2^0$ and $[\theta]_{222}^{100}$,

corresponding to 0% and 100% helical contents at 222 nm, were estimated to be −2000 and −30 000 deg cm^2/dmol, respectively.

RESULTS AND DISCUSSION

The purities of the synthetic peptides were more than 95%, as assessed by the elution profile in analytical RP-HPLC. The correct amino acid compositions of the peptides were confirmed by amino acid analysis (data not shown). The sequences of the synthetic peptides are shown in Table 1. The correct molecular weights of the synthetic peptides were confirmed by MALDI mass spectrometry (Table 2).

The antiviral activities of the peptides were determined by a syncytia formation inhibition assay. The values of antiviral activities of the peptides are illustrated in Table 3.

The amphipathic feature of α-helical antimicrobial peptides plays an important role against target cells. A number of parameters including net positively charge, α-helicity and overall hydrophobicity have been shown to modulate the antibiotic activity of the α-helical amphipathic antimicrobial peptides [22]. In our previous study, the analogue peptide, L^{16}-CA(1–8)-MA(1–12), with Leu substitution of Ser at position 16 in CA(1–8)-MA(1–12) was

Table 1 Amino Acid Sequences of the Analogues with Amino Acid Substitution at Position 16 in CA(1–8)-MA(1–12)

Peptide	Amino acid sequence
CA(1–8)-MA(1–12)	KWKLFKKIGIGKFLHSAKKF-NH$_2$
A^{16}-CA(1–8)-MA(1–12)	KWKLFKKIGIGKFLHAAKKF-NH$_2$
V^{16}-CA(1–8)-MA(1–12)	KWKLFKKIGIGKFLHVAKKF-NH$_2$
F^{16}-CA(1–8)-MA(1–12)	KWKLFKKIGIGKFLHFAKKF-NH$_2$
W^{16}-CA(1–8)-MA(1–12)	KWKLFKKIGIGKFLHWAKKF-NH$_2$

Table 2 Molecular Weights of the Synthetic Peptides Determined by MALDI-MS

Peptide	Calculated value	Observed value	Retention time (min)
CA(1–8)-MA(1–12)	2402.48	2403.0	17.453
A^{16}-CA(1–8)-MA(1–12)	2386.49	2387.0	17.779
V^{16}-CA(1–8)-MA(1–12)	2428.53	2429.0	18.849
F^{16}-CA(1–8)-MA(1–12)	2462.52	2463.0	19.250
W^{16}-CA(1–8)-MA(1–12)	2501.53	2502.0	19.522

Table 3 Antiviral Activities of CA-MA and its Analogue Peptides

Peptide	Fusion index (FI)	% Fusion inhibition
CA(1–8)-MA(1–12)	0.37 ± 0.01	45.27 ± 2.02
A^{16}-CA(1–8)-MA(1–12)	0.34 ± 0.08	49.86 ± 11.74
V^{16}-CA(1–8)-MA(1–12)	0.25 ± 0.03	63.21 ± 4.41
F^{16}-CA(1–8)-MA(1–12)	0.21 ± 0.06	69.30 ± 8.25
W^{16}-CA(1–8)-MA(1–12)	0.13 ± 0.01	81.17 ± 1.34
Virus control	0.68 ± 0.06	0

observed to show more potent antifungal activity than CA(1–8)-MA(1–12) [16]. These results suggest that the hydrophobicity at position 16 plays an important role in the antimicrobial activities of CA(1–8)-MA(1–12). However, the antiviral effect of the amino acid residue at position 16 in CA(1–8)-MA(1–12) has not yet been reported. In order to investigate the effects of hydrophobicity of the

residue at position 16 in the antiviral activity of CA(1–8)-MA(1–12), A^{16}-CA(1–8)-MA(1–12), V^{16}-CA(1–8)-MA(1–12), F^{16}-CA(1–8)-MA(1–12) and W^{16}-CA(1–8)-MA(1–12) were synthesized (Table 1). In this study, these analogue peptides, except A^{16}-CA(1–8)-MA(1–12), showed a potent antiviral activity compared with CA(1–8)-MA(1–12). As position 16 is located in a hydrophobic face as shown in the α-helical wheel diagram of CA(1–8)-MA(1–12) (Figure 1), the increase of hydrophobicity at this position caused a significant increase in antiviral activity. As shown in Figure 2, the peptide inhibited the syncytia formation derived from $CD4^{+}$ HeLa cells and vPE 16. The order of virus–cell fusion inhibitory activity of the synthetic peptides used in this study was W^{16}-CA(1–8)-MA(1–12) > F^{16}-CA(1–8)-MA(1–12) > V^{16}-CA(1–8)-MA(1–12) > A^{16}-CA(1–8)-MA(1–12) > CA(1–8)-MA(1–12). These results suggest that the hydrophobicity at position 16 of the peptide is related to antiviral activity.

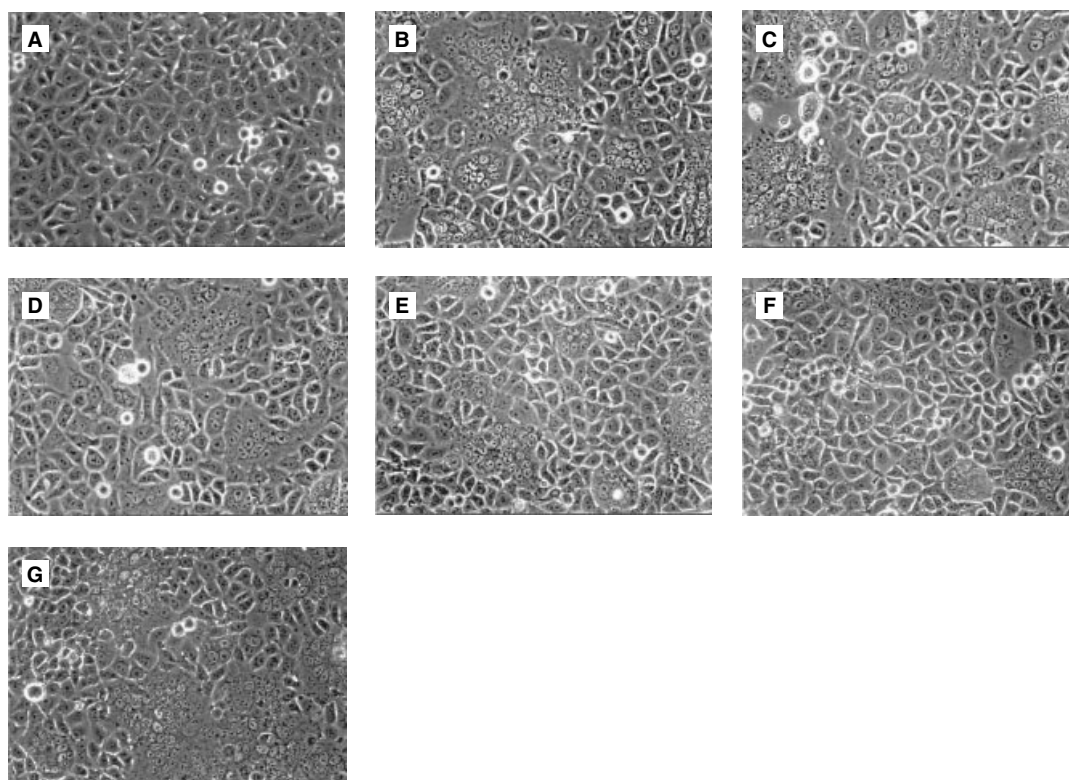In order to investigate the relationship of the structure and the antiviral activity of the peptides

Figure 2 Visualization of virus–cell fusion inhibitory of CA(1–8)-MA(1–12) and its analogue peptides. HeLa/$CD4^{+}$ cells (CD4 receptor encoding HeLa S3 cells) vPE 16 (HIV envelope protein gp 120/41 encoding vaccinia virus) Syncytia formation inhibition assay. A: Cell control; B: CA(1–8)-MA(1–12); C: A^{16}-CA(1–8)-MA(1–12); D: V^{16}-CA(1–8)-MA(1–12); E: F^{16}-CA(1–8)-MA(1–12); F: W^{16}-CA(1–8)-MA(1–12); G: Virus control.

Table 4   Analysis of CD Spectra for Secondary Structure Content of CA(1–8)-MA(1–12) and its Analogue

Peptide	Buffer		SDS (30 mM)		TFE (50%)	
	$[\theta]_{222}$[a]	% α-Helix[b]	$[\theta]_{222}$	% α-Helix	$[\theta]_{222}$	% α-Helix
CA(1–8)-MA(1–12)	−5367	0.79	−88296	12.9	−238909	35.0
A^{16}-CA(1–8)-MA(1–12)	−5590	0.82	−118913	17.4	−233179	34.1
V^{16}-CA(1–8)-MA(1–12)	−1994	0.29	−110593	16.2	−226313	33.1
F^{16}-CA(1–8)-MA(1–12)	−7884	1.15	−117102	17.1	−241975	35.4
W^{16}-CA(1–8)-MA(1–12)	−4491	0.66	−128047	18.7	−219961	32.2

[a] Mean residue ellipticity is expressed in deg cm^2/dmol.
[b] Calculated by the equation: % helicity $= 100([\theta]_{222} - [\theta]_2^0)/[\theta]_{222}^{100}$ where θ is the experimentally observed mean residue ellipticity at 222 nm. Values for $[\theta]_2^0$ and $[\theta]_{222}^{100}$, corresponding to 0% and 100% helical contents at 222 nm, were estimated to be −2000 and −30000 deg cm^2/dmol, respectively.

on lipids, the CD spectra of the peptides in phosphate buffer and TFE solution or SDS micelles were measured. CA(1–8)-MA(1–12) showed similar α-helical contents to the other analogue peptides in 50% TFE solution. In contrast, although W^{16}-CA(1–8)-MA(1–12) has more potent virus–cell fusion inhibitory activity than CA(1–8)-MA(1–12), it displayed similar α-helicity to CA(1–8)-MA(1–12) (Table 4). These results suggest that the α-helical content is not directly correlated with the enhanced antiviral activity even though it may play an important role in virus cells.

CONCLUSION

In order to investigate the relationships between the peptide structure and antiviral activity of CA(1–8)-MA(1–12), A^{16}-CA(1–8)-MA(1–12), V^{16}-CA(1–8)-MA(1–12), F^{16}-CA(1–8)-MA(1–12) and W^{16}-CA(1–8)-MA(1–12) were synthesized. In particular, substitution of Ser with a hydrophobic amino acid, Val, Phe or Trp at position 16 caused a dramatic increase in virus–cell fusion inhibitory activity.

Acknowledgements

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