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Structure–activity Relationship of an Antibacterial Peptide, Maculatin 1.1, from the Skin Glands of the Tree Frog, *Litoria genimaculata*

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Abstract: Maculatin 1.1 (Mac) is a cationic antibacterial peptide isolated from the dorsal glands of the tree frog, *Litoria genimaculata*, and has a sequence of GLFGVLAKVAAHVVPAIAEHF-NH_2. A short peptide lacking the *N*-terminal two residues of Mac was reported to have no activity. To investigate the structure–activity relationship in detail, several analogs and related short peptides of Mac were synthesized. CD measurement showed that all the peptides took more or less an α-helical structure in the presence of anionic lipid vesicles. Analogs which are more basic than Mac had strong antibacterial and hemolytic activities, while short peptides lacking one or two terminal residues exhibited weak or no activity. Outer and inner membrane permeabilization activities of the peptides were also reduced with shortening of the peptide chain. These results indicate that the entire chain length of Mac is necessary for full activity, and the basicity of the peptides greatly affects the activity. Copyright © 2004 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: maculatin 1.1; antibacterial peptide; hemolytic activity; membrane permeabilization

INTRODUCTION

Cationic amphiphilic peptide antibiotics have been found from a variety of natural sources, and they have been designed artificially in the search for desirable candidates for clinical use or to clarify the structure–activity relationship [1–6]. In this connection, much attention has been concentrated on finding peptides with strong antibacterial activity but with no hemolytic activity [7–10], because hemolytic activity is undesirable for practical use of the peptides as drugs.

Maculatins are peptides isolated from the dorsal glands of the tree frog, *Litoria genimaculata* [11]. A few of them showed antibacterial activity, and a peptide named maculatin 1.1 (Mac) had the strongest activity against Gram-positive bacteria and Gram-negative bacteria. Mac consists of 21 amino acid residues and was supposed to become amphiphilic when it takes an α-helical structure (Figure 1). The lack of the *N*-terminal two residues from Mac led to the loss of antibacterial activity completely [11]. By

Abbreviations: CD, circular dichroism; Mac, maculatin 1.1; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; MIC, minimum inhibitory concentration; NPN, *N*-phenyl-1-naphthylamine; ONPG, *o*-nitrophenyl β-galactopyranoside; PBS, phosphate buffered saline (pH 7.5); PC, 3-phosphatidylcholine; PG, phosphatidylglycerol; RP-HPLC, reversed phase high-performance liquid chromatography; SUV, small unilamellar vesicle; TFE, 2,2,2-trifluoroethanol; TSB, tryptic soy broth.

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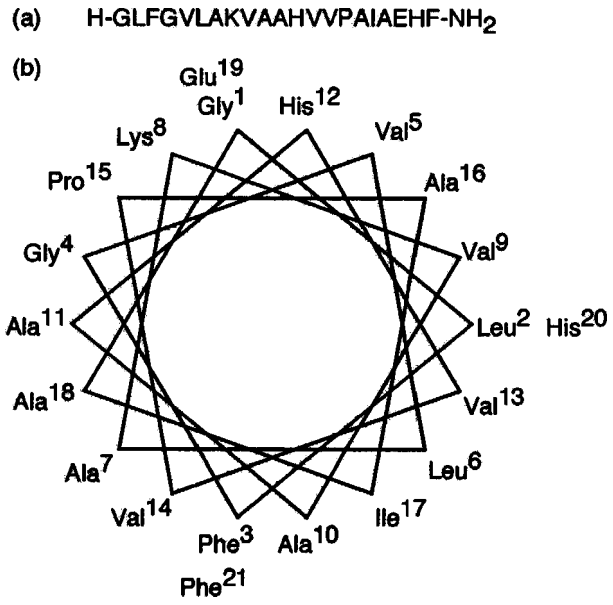

Figure 1 Amino acid sequence of Mac (a) and its helical wheel (b).

comparing Mac with another antibacterial peptide, caerin 1.1, of which the structure–activity relationship was previously investigated [12–14], it was pointed out that the Pro^{15} residue in Mac might play an important role in antibacterial activity. However, there has been no detailed study on the structure–activity relationship of Mac. In this study, Mac and the related peptides were synthesized to clarify the relationship. The synthetic peptides are the Mac analogs with one or two more Lys residues than Mac, [A^{15}]Mac has Ala instead of Pro^{15} in Mac, and several short peptides which are devoid of one or two terminal residues of Mac. The properties of these peptides were examined by CD and calcein-leakage measurements, and their biological activities were evaluated by antibacterial, hemolytic and bacterial membrane permeabilization assays. The peptides having high α-helicity and strong calcein-leakage activity generally showed strong biological activities. The entire chain length of Mac was found to be necessary for full biological activity.

MATERIALS AND METHODS

Fmoc-amino acids, 4-(2′,4′-dimethoxyphenylaminomethyl)phenoxy resin (Rink Amide resin) and the coupling reagents were purchased from Watanabe Chemical Industries Ltd (Hiroshima) and Calbiochem-Novabiochem AG (Switzerland). Egg PC and egg PG were from Funakoshi Co. Ltd (Tokyo). Calcein was from Dojindo Laboratories (Kumamoto). Rabbit blood and bacteria were from Nippon Biotest Laboratries (Tokyo) and Fermentation Institute for Fermentation, Osaka (IFO) (Osaka), respectively. *N*-phenyl-1-naphthylamine (NPN) was from Wako Pure Chemical Industries Ltd (Osaka).

Peptide Synthesis

All the peptides were synthesized by the solid-phase method using Fmoc-amino acids and Rink Amide resin with a Shimadzu PSSM-8 peptide synthesizer. The products were purified by gel chromatography on a Sephadex G-10 column followed by RP-HPLC on a YMC-Pack ODS-A column. The purity of the final products was evaluated by analytical RP-HPLC, and was found to exceed 95%. Characterization of the products was performed by MALDI-TOF-MS and amino acid analysis.

Preparation of Phospholipid Vesicles

The SUVs of egg PC and egg PC/egg PG (3 : 1) were prepared for the CD and spectrophotometric measurements. Phospholipid (5 μmol) was dissolved in $CHCl_3$/MeOH (2 : 1 v/v, 0.6 ml), then dried under a stream of N_2 gas. The dried lipid was hydrated in 20 mM Tris HCl buffer (pH 7.4, 5 ml) using a Branson bath-type sonicator. The suspension was sonicated for 10 min at 25 °C using a Taitec ultrasonic processor VT-5T at an intensity of 10 W. The vesicles were allowed to stand for 30 min at 25 °C before the measurements were made. The lipid concentration was 1 mM. Calcein-entrapped vesicles were similarly prepared. The dried egg PC/egg PG (3 : 1) (5 μmol) was hydrated in 20 mM Tris HCl buffer (pH 7.4, 2 ml) containing 60 mM calcein and the suspension was sonicated as described above. The calcein-entrapped vesicles were separated from the free calcein by gel filtration using Sephadex G-75 (1.1 × 27 cm) with the same buffer (pH 7.4). The fraction (4 ml) containing calcein-entrapped vesicles was pooled.

Circular Dichroism Measurements

The CD spectra were recorded on a Jasco J-720 W spectropolarimeter with a thermostatted cell holder using a quartz cell of 1.0 mm path length. The peptides were dissolved in the buffer (pH 7.4), TFE and buffer containing 1 mM phospholipid vesicles. The peptide concentration was 20 μM.

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Measurements were done at 25 °C. The mean residue ellipticity is given as deg cm^2 $dmol^{-1}$ and the α-helical contents were calculated according to the method of Scholtz *et al.* [15].

Antibacterial and Hemolytic Activity

The serial solution dilution method was used to determine the MIC values as described by Yoshida *et al.* [16]. The cell suspension was diluted with TSB medium (pH 7.4) to 10^4 cells/ml. Several concentrations of the peptide solution were placed in the test tubes, made up to 20 µl with medium and cell suspension (180 µl) was added. After incubation for 24 h at 37 °C, absorbance at 620 nm was measured. Hemolytic activity was assayed by the method of Yoshida *et al.* [16]. PBS (pH 7.5, 0.5 ml) was added to fresh rabbit blood (0.5 ml). The resulting mixture was centrifuged at 2000 rpm for 3 min, and the precipitates were collected. After being washed with PBS three times, the obtained precipitates were suspended in PBS (2 ml). PBS (1 ml) was added to the rabbit erythrocyte solution (5 µl) followed by the peptide solution. The resulting suspension was incubated for 20 min at 37 °C, and then centrifuged. The supernatant was monitored at 413 nm on a Hitachi U-2000 spectrophotometer. To measure the absorbance for 100% activity, 10% Triton X-100 (10 µl) instead of peptide was added to the erythrocyte solution.

Calcein Leakage

The calcein leakage experiment was performed by the procedure of Lee *et al.* [17]. The vesicles (10 µl) containing 60 mM calcein was mixed with 20 mM Tris HCl buffer (pH 7.4, 1 ml) in a cuvette, resulting in a final phospholipid concentration of 10 µM. While the cuvette was in the holder of a Hitachi F-3010 spectrofluorometer at 25 °C, 20 µl of the appropriate dilution of a peptide solution in buffer was added to the cuvette within 10 mm path length. The fluorescence intensities of calcein were monitored at 515 nm (excited at 495 nm) and measured 2 min after adding the peptide. To measure the fluorescence intensity corresponding to 100% calcein release, 10% Triton X-100 (10 µl) was added to dissolve the vesicles. The percentage of the calcein release was calculated by the equation $100 \times (F - F_0)/(F_t - F_0)$, where F is the intensity observed by adding the buffer instead of a peptide solution, and F_t is the intensity observed after Triton X-100 treatment.

Outer Membrane Permeabilization

Determination of the outer membrane permeability was performed by measuring the fluorescence intensity of NPN as described by Wu *et al.* [18]. Single colonies of *E. coli* grown on trypticase soy agar plates were inoculated in TSB (5 ml) and grown overnight at 37 °C. The suspension was diluted with TSB to an absorbance of 0.5–0.6 at 600 nm. Then 100 µl of 320 µM peptide solution was added to a mixture of the bacterial suspension (880 µl) and 0.5 mM NPN in acetone (20 µl). The outer membrane permeability was monitored by measuring the intensity at 420 nm (excited at 350 nm) for 10 min at 25 °C. An equivalent volume of water was used instead of the peptide solution in the control assay.

Inner Membrane Permeabilization

Determination of the inner membrane permeability was performed by measuring the galactosidase activity using ONPG as a substrate according to the procedure of Pellegrini *et al.* [19]. A suspension of *E. coli* (10^4 cells/ml, 880 µl) was added to a solution of 2.5 mM ONPG in TSB medium (pH 7.4, 20 µl). After 15 min of incubation, 320 µM peptide solution (100 µl) was added. The inner membrane permeability was monitored by measuring the rate of *o*-nitrophenol production at 420 nm every 1 min. An equivalent volume of water was used instead of the peptide solution in the control assay.

RESULTS AND DISCUSSION

Design and Synthesis of Peptides

The balance between cationic and hydrophobic amino acid residues is generally an important factor for antibacterial activity [20]. When Mac takes an α-helix structure, the Lys^8, His^{12} and Glu^{19} residues are in the same direction (Figure 1) [7]. Coexistence of cationic and anionic residues in the same direction would be disadvantageous for antibacterial activity because of the cancellation of charge. To examine the effect of a change of basicity of Mac on antibacterial activity, three 21mer analogs, [K^{12}]Mac, [K^{19}]Mac and [$K^{12,19}$]Mac, were designed. Rozek *et al.* pointed out that Pro in Mac seemed to play an important role in antibacterial activity [11]. Lee *et al.* also reported that substitution of a Pro residue by other amino acids in a model peptide caused a change in biological activity [21]. Therefore,

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[A^{15}]Mac, which has an Ala^{15} residue instead of Pro in Mac, was designed. The peptide lacking the *N*-terminal two residues of Mac was found to be inactive [11]. This finding suggested that the full peptide chain length, appropriate α-helicity and/or hydrophobicity would be required for antibacterial activity. On the basis of such an assumption, several short peptides lacking one or two residues from the *N*- or *C*-terminus of Mac were designed. The structures of the designed peptides are shown in Table 1. The peptides were synthesized by the solid-phase method using Fmoc-amino acids and Rink Amide resin in the usual manner. After purification by gel chromatography followed by RP-HPLC, the final products were identified by MALDI-TOF-MS (Table 1) and amino acid analysis (data not shown).

Circular Dichroism

CD measurements were performed in 20 mM Tris HCl buffer (pH 7.4), TFE, and the buffer in the presence of egg PC or egg PC/egg PG (3 : 1) SUVs (Table 2). In the buffer, Mac and the 21mer analogs other than [A^{15}]Mac had low α-helical contents of 6%–8%, while the α-helical content of [A^{15}]Mac was 25%. This result suggested that replacement of Pro^{15} by other amino acids might change the conformation and nature of Mac. Although [Δ^1]Mac showed an α-helical content of 6%, other short peptides were random in aqueous environment. In TFE, an α-helix-inducing solvent, all the peptides exhibited α-helical curves with double minima around 208 and 222 nm, corresponding to an α-helical content of 26%–50%. Mac and the analogs have moderate α-helicities, while the helical contents of the short peptides are generally low.

A similar tendency in the α-helical contents of the 21mer peptides and the short peptides was observed in the presence of egg PC. The considerably lower α-helicity of the short peptides suggests that they might not be able to sufficiently interact with neutral egg PC vesicles. All the peptides including the short peptides had higher α-helical contents in the presence of egg PC/egg PG (3 : 1) vesicles than in the presence of egg PC vesicles. These results indicate that these cationic peptides interacted more strongly with anionic phospholipid vesicles by a charge interaction in addition to hydrophobic interaction. The lower α-helicity of [Δ^1]Mac than that of Mac implied that the entire chain length of Mac might be important or necessary for biological activity. In the case of [A^{15}]Mac the exact α-helical content could not be determined, because of formation of some turbidity after mixing the peptide with neutral or anionic phospholipid vesicles.

Antibacterial and Hemolytic Activities

The antibacterial activity of the peptides was examined by the serial solution dilution method using two Gram-positive bacteria (*S. aureus* and *B. subtilis*) and two Gram-negative bacteria (*E. coli* and *P. aeruginosa*). Gramicidin S, a natural antibacterial peptide, was used as a reference. The MIC values

Table 1 Amino Acid Sequences, Molecular Weights and Hydrophobicity Values of Peptides

Peptide	Amino acid sequence	MALDI-TOF-MS		$\langle H \rangle$[a]
		Found	Calcd $[M + H]^+$	
Mac	H-GLFGVLAKVAAHVVPAIAEHF-NH_2	2146.2	2145.6	0.198
[K^{12}]Mac	H-GLFGVLAKVAAKVVPAIAEHF-NH_2	2137.3	2136.6	0.164
[K^{19}]Mac	H-GLFGVLAKVAAHVVPAIAKHF-NH_2	2145.1	2144.6	0.175
[K^{12,19}]Mac	H-GLFGVLAKVAAKVVPAIAKHF-NH_2	2135.6	2135.7	0.141
[A^{15}]Mac	H-GLFGVLAKVAAHVVAAIAEHF-NH_2	2120.1	2119.6	0.213
[Δ^1]Mac	H-LFGVLAKVAAHVVPAIAEHF-NH_2	2089.3	2088.6	0.200
[Δ^1,A^2]Mac	H-AFGVLAKVAAHVVPAIAEHF-NH_2	2047.4	2046.5	0.186
[Δ^1,G^2]Mac	H-GFGVLAKVAAHVVPAIAEHF-NH_2	2033.3	2032.4	0.181
[$\Delta^{1,2}$]Mac	H-FGVLAKVAAHVVPAIAEHF-NH_2	1976.3	1975.4	0.182
[Δ^{21}]Mac	H-GLFGVLAKVAAHVVPAIAEH-NH_2	1998.4	1998.4	0.177
[$\Delta^{20,21}$]Mac	H-GLFGVLAKVAAHVVPAIAE-NH_2	1861.9	1861.3	0.207

[a] Hydrophobicity/residue. Hydrophobicity was calculated using the consensus value of hydrophobicity value for each amino acid residue [36].

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Table 2 α-Helical Contents and Antibacterial Activities of Peptides

Peptide	α-Helical content (%)[a]				MIC (μM)[g]			
	Buffer[b] (pH 7.4)	98%TFE	Egg PC[c]	Egg PC/egg PG[c] (3 : 1)	*S. aureus* IFO 12732	*B. subtilis* IFO 3134	*E. coli* IFO 12734	*P. aeruginosa* IFO 12582
Mac	8	43	36	44	8	8	32	64
[K^{12}]Mac	8	40	36	47	8	8	16	32
[K^{19}]Mac	7	36	32	45	4	4	16	32
[$K^{12,19}$]Mac	8	43	39	51	4	2	8	16
[A^{15}]Mac	25	50	[25][d]	[25][d]	4	8	>64	>64
[Δ^1]Mac	6	32	25	30	>64	>64	>64	>64
[Δ^1,A^2]Mac	R[e]	29	16	32	>64	>64	>64	>64
[Δ^1,G^2]Mac	R[e]	26	12	30	>64	>64	>64	>64
[$\Delta^{1,2}$]Mac	R[e]	27	10	29	>64	>64	>64	>64
[Δ^{21}]Mac	R[e]	35	19	36	>64	>64	>64	>64
[$\Delta^{20,21}$]Mac	R[e]	29	12	33	>64	>64	>64	>64
Gramicidin S	—[f]	—[f]	—[f]	—[f]	4	2	16	32

[a] [Peptide] = 20 μM, 25 °C.
[b] 20 mM Tris HCl buffer (pH 7.4).
[c] [Lipid] = 0.98 mM.
[d] Exact α-helical contents were not able to be determined because of turbidity.
[e] R means random.
[f] Not determined.
[g] Method, serial solution dilution method; medium, TSB medium (pH 7.4); inoculum size, 10^4 cells/ml. After incubation for 24 h at 37 °C, absorbance at 620 nm was measured.

of the peptides are listed in Table 2. Mac, [K^{12}]Mac, [K^{19}]Mac and [$K^{12,19}$]Mac were active against all the bacteria used, and their antibacterial activities were found to be stronger against Gram-positive bacteria than Gram-negative bacteria. The activity increased with the increase in the Lys residue, and [$K^{12,19}$]Mac exhibited higher activity than gramicidin S against Gram-negative bacteria. Replacement of Glu^{19} by Lys induced a clear increase in the activity, while the effect of the $His^{12} \rightarrow$ Lys substitution on the activity was small. In this connection, Mihara *et al.* previously reported that introduction of a cationic amino acid in amphiphilic α-helical peptides strengthened antibacterial activity against Gram-negative bacteria [22]. However, an increase in antibacterial activities of the Lys-containing analogs was found against both Gram-positive bacteria and Gram-negative bacteria in this study. It is noticeable that the $Pro^{15} \rightarrow$ Ala substitution maintained the strong antibacterial activity against Gram-positive bacteria but lost the activity against Gram-negative bacteria, suggesting that a long helical structure is disadvantageous for Gram-negative bacteria. It was previously found that the $Ala^6 \rightarrow$ Pro substitution in Ac-(Leu-Ala-Arg-Leu)$_3$-$NHCH_3$ caused a decrease in antibacterial activity against Gram-positive bacteria, but a retention or slight increase in the activity against Gram-negative bacteria [21]. [Δ^1]Mac, [Δ^1,A^2]Mac, [Δ^1,G^2]Mac and [$\Delta^{1,2}$]Mac were inactive against all the bacteria. The result that [$\Delta^{1,2}$]Mac showed no activity agreed with the data reported by Rozek *et al.* [11]. The hydrophobicities of [Δ^1,A^2]Mac, [Δ^1,G^2]Mac and [$\Delta^{1,2}$]Mac were lower than those of Mac and [Δ^1]Mac, suggesting that higher hydrophobicity seems to be favorable for the activity. However, [$\Delta^{20,21}$]Mac was devoid of activity in spite of its high hydrophobicity, and the Lys-containing analogs with low hydrophobicity had strong activity. It is likely that amphiphilicity, i.e. a sharp separation of the cationic cluster from the hydrophobic cluster in the peptide molecule, is essential for the activity. Furthermore, a certain peptide chain length may be required for retention of the biologically active conformation of Mac.

Cationic antibacterial peptides often have hemolytic activity [23–25]. From the point of view of the practical use as a drug, it is desirable to find antibacterial peptides without hemolytic activity [7,8,26]. Therefore, the hemolytic activity of the peptides was examined using rabbit red blood

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cells [16]. A part of the result is shown Figure 2. Mac and the 21mer analogs had fairly strong hemolytic activities. The hemolytic activity of Mac was considerably stronger compared with that of magainin 2, a well-known natural antibacterial peptide with weak hemolytic activity [24]. This may be due to the high hydrophobicity of Mac; the $< H >$ values of Mac and magainin 2 are 0.198 and −0.036, respectively. Among the 21mer analogs, the highest hemolytic activity was observed for [A^{15}]Mac. Since the differences between Mac and [A^{15}]Mac are α-helicity and hydrophobicity, α-helicity may also contribute, in addition to hydrophobicity, to hemolytic activity. [Δ^1]Mac showed weak activity, while other 20mer and 19mer peptides were inactive even at a peptide concentration of 100 μM. The finding that Mac has hemolytic activity indicates that some skilful changes in the α-helicity and hydrophobicity of Mac will be required to convert Mac to a nonhemolytic derivative.

There have been many studies on the effects of the Pro → Ala substitution in melittin [27–29] and alamethicin [30–32] on biological activities and structural properties. [A^{14}]melittin exhibited stronger membrane-binding affinity [27] and hemolytic activity compared with melittin [29]. These results agree with the present data. The different features of the analogs from melittin were concluded to be ascribable to structural changes caused by helix stabilization [27,28]. This may also be the case for [A^{15}]Mac, because its helicity is fairly different from that of Mac. Furthermore, analytical studies on the structure–activity relationship indicate that Pro-induced kinks are important in the biological activities of antibacterial properties and Pro substitution often results in a remarkable change of action [30–34].

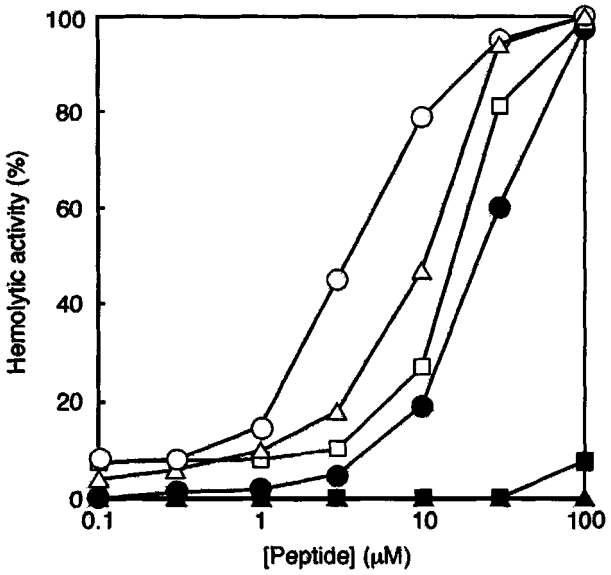

Figure 2 Profiles of hemolysis as a function of the peptide concentration for rabbit red blood cells. Incubation; PBS (pH7.5), 20 min, 37 °C. Mac (●), [K^{19}]Mac (□), [$K^{12,19}$]Mac (Δ), [A^{15}]Mac (○), [Δ^1]Mac (■) and [$\Delta^{1,2}$]Mac (▲).

Calcein Leakage

The bacterial cell membrane is the locus of action for many cationic antibacterial peptides [1,4,35]. Calcein-leakage activity of the peptides was measured to confirm that the peptides could interact with the phospholipid bilayer mimicking the bacterial cell membrane. A part of the result is shown in Figure 3. [K^{12}]Mac, [K^{19}]Mac and [$K^{12,19}$]Mac exhibited strong calcein leakage activity in the presence of egg PC/egg PG (3 : 1) vesicles, and Mac also showed fairly strong activity. The leakage activity gradually decreased with the stepwise deletion of the *N*- and *C*-terminal amino acid residues. This result is compatible with that of antibacterial activity and therefore suggests that the target site for the antibacterial action of Mac is the bacterial cell membrane. Short peptides which are biologically inactive unexpectedly exhibited leakage

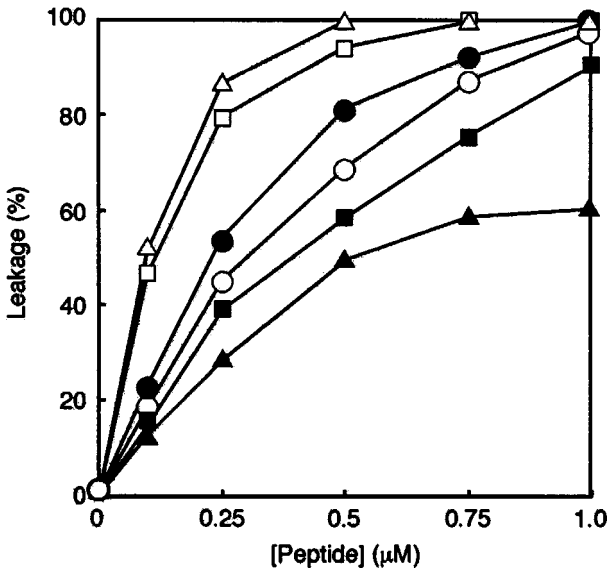

Figure 3 Calcein-leakage activities of the peptides for egg PC/egg PG (3 : 1) SUVs. [Lipid] = 10 μM, 25 °C, λex = 495 nm and λem = 515 nm. Leakage was measured 2 min after adding the peptide. Mac (●), [K^{19}]Mac (□), [$K^{12,19}$]Mac (Δ), [A^{15}]Mac (○), [Δ^1]Mac (■) and [$\Delta^{1,2}$]Mac (▲).

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activity. This may be because artificial anionic vesicles are very sensitive to cationic peptides compared with the bacterial cell membrane.

Outer and Inner Membrane Permeability

NPN, a hydrophobic fluorescent probe, penetrates into the membrane interior when the membrane is disturbed. Since the fluorescence intensity increases in a hydrophobic environment, it is applicable to permeabilization measurement. The membrane permeabilization activity of the peptides is related strongly to their antibacterial activity. Therefore, permeabilization activities of the peptides were examined with the outer membrane of *E. coli* (Figure 4). Mac and [Δ^1]Mac exhibited moderate permeabilization activities, while [$\Delta^{1,2}$]Mac had negligible activity. The permeabilization activity of [K^{19}]Mac was higher than that of Mac. These results are reasonable, because the order of permeabilization activities of the peptides is in accord with that of their antibacterial activities. However, [A^{15}]Mac unexpectedly showed the strongest permeabilization activity among the peptides, although its antibacterial activity was negative against *E. coli*. Although it is hard to explain this phenomenon, a possibility is that [A^{15}]Mac may exhibit biological activity not by membrane perturbation but by an other mechanism, e.g. action on a target other than the cell membrane.

The inner membrane permeabilization was evaluated by measuring β-galactosidase activity using ONPG as a substrate [19]. When the permeabilization of the inner membrane is increased by the peptide, ONPG moves into the cytoplasm and is hydrolysed by the enzyme to produce *o*-nitrophenol, which is colored under weakly alkaline conditions. Absorbance at 420 nm rapidly increased with time (Figure 5), where gramicidin S was used as a reference. The order of permeabilization activities of the peptides was [$K^{12,19}$]Mac > Mac > [Δ^1]Mac. This result is again consistent with that of their antibacterial activities and outer membrane permeabilization activities. Gramicidin S exhibited the strongest antibacterial activity and membrane permeabilization activity, suggesting that high hydrophobicity and sharp separation of the charged and hydrophobic side chains are favorable for biological activity. Since the fluorescence intensity rapidly increased and reached a plateau afterwards, the main change of the inner membrane structure might occur at an early time.

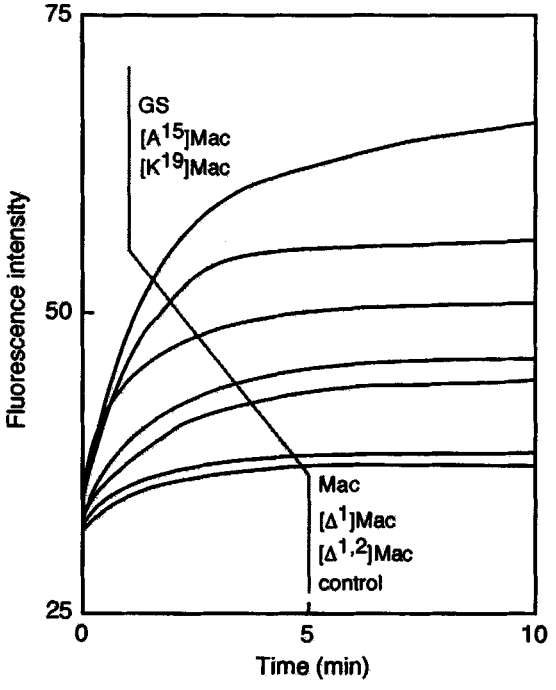

Figure 4 Outer membrane permeabilization activities of the peptides assessed by the NPN uptake in *E. coli*. [Peptide] = 32 μM, [NPN] = 10 μM, λex = 350 nm and λem = 420 nm.

CONCLUSION

To examine the structure–activity relationship of Mac, several analogs were prepared of Mac and its short peptides lacking *N*- or *C*- terminal residue(s). It was found that a lack of the terminal residue(s)

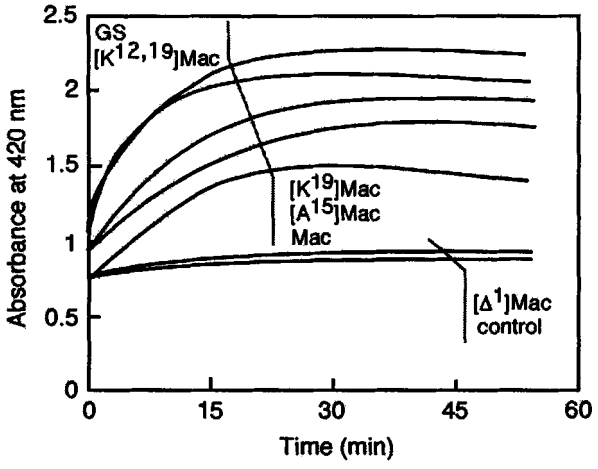

Figure 5 Inner membrane permeabilities of the peptides assessed by the ONPG uptake in *E. coli*. [Peptide] = 32 μM and [ONPG] = 50 μM.

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greatly reduced the binding ability of the peptides to the lipid membrane and their biological activities, that is, the entire peptide chain was necessary for full activity and keeping an active conformation. The introduction of Lys residue(s) in Mac caused an increase in antibacterial and hemolytic activities, while the Pro → Ala substitution enhanced hemolytic activity. These results indicate that cationic residues mainly participate in antibacterial activities, and hydrophobic residues affect both antibacterial and hemolytic activities. A consideration of cationic residues and hydrophobic residues is required in the design of the desirable peptides with strong antibacterial activity but less or no hemolytic activity. Information from the present work would be useful for the investigations on the structure–activity relationship of Mac-type peptides.

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REFERENCES

1. Saberwal G, Nagaraj R. Cell-lytic and antibacterial peptides that act by perturbing the barrier function of membranes: facets of their conformational features, structure-function correlations and membrane-perturbing activities. *Biochim. Biophys. Acta* 1994; **1197**: 109–131.
2. Tossi A, Sandri L, Giangaspero A. Amphipathic, α-helical antimicrobial peptides. *Biopolymers (Peptide Sci.)* 2000; **55**: 4–30.
3. Deber CM, Li S-C. Peptides in membranes: helicity and hydrophobicity. *Biopolymers (Peptide Sci.)* 1995; **37**: 295–318.
4. Shai Y, Oren Z. From 'carpet' mechanism to *de-novo* designed diasteromeric cell-selective antimicrobial peptides. *Peptides* 2001; **22**: 1629–1641.
5. Dathe M, Meyer J, Beyerman M, Maul B, Hoischen C, Bienert M. General aspect of peptide selectivity towards lipid bilayers and cell membranes studied by variation of the structural parameters of amphiphilic helical model peptides. *Biochim. Biophys. Acta* 2002; **1558**: 171–186.
6. Hancock RE. Peptide antibiotics. *Lancet* 1997; **349**: 418–422.
7. Park CB, Lee JH, Park IY, Kim MS, Kim SC. A novel antimicrobial peptides from the loach, *Misgurnus auguillicaudatus*. *FEBS Lett.* 1997; **411**: 173–178.
8. Tam JP, Lu Y-A, Yang J-L. Antimicrobial dendritic peptides. *Eur. J. Biochem.* 2002; **269**: 923–932.
9. Stark M, Liu LP, Deber CM. Cationic hydrophobic peptides with antimicrobial activity. *Antimicrob. Agents Chemother.* 2002; **46**: 3585–3590.
10. Park Y, Lee DG, Jang S-H, Woo E-R, Jeong HG, Choi C-H, Hahm K-S. A Leu-Lys-rich antimicrobial peptide: activity and mechanism. *Biochim. Biophys. Acta* 2003; **1645**: 172–182.
11. Rozek T, Waugh RJ, Steinborner ST, Bowie JH, Tyler MJ, Wallace JC. The maculatin peptides from the skin glands of the tree frog *Litoria genimaculata*: a comparison of the structures and antibacterial activities of maculatin 1.1 and caerin 1.1. *J. Peptide Sci.* 1998; **4**: 111–115.
12. Stone DJM, Waugh RJ, Bowie JH, Wallace JC, Tyler MJ. The structures of the caerins and caeridin 1 from *Litoria splendida*. *J. Chem. Soc. Perkin Trans. 1.* 1992; 3173–3178.
13. Stone DJM, Waugh RJ, Bowie JH, Wallace JC, Tyler MJ. Structures of the caerins from *Litoria caeulea*. *J. Chem. Res. (C)* 1993; **138**(M): 910–936.
14. Waugh RJ, Stone DJM, Bowie JH, Wallace JC, Tyler MJ. The structures of the caerins and caeridins from *Litoria gillent*. *J. Chem. Res. (M)* 1993; **139**(M): 937–961.
15. Scholtz JM, Quian H, York EJ, Stewart JM, Baldwin RL. Parameters of helix-coil transition theory for alanine based peptides of varying chain lengths in water. *Biopolymers* 1991; **31**: 1463–1470.
16. Yoshida K, Mukai Y, Niidome T, Takashi C, Tokunaga Y, Hatakeyama T, Aoyagi H. Interaction of pleurocidin and its analogs with phospholipids membrane and their antibacterial activity. *J. Peptide Res.* 2001; **57**: 119–126.
17. Lee S, Mihara H, Aoyagi H, Kato T, Izumiya N, Yamasaki N. Relationship between antimicrobial activity and amphiphilic property of basic model peptides. *Biochim. Biophys. Acta* 1986; **862**: 211–219.
18. Wu M, Hancock REW. Interaction of cyclic antibacterial cationic peptide bactenecin with the outer and cytoplasmic membrane. *J. Biol. Chem.* 1999; **274**: 29–35.
19. Pellegrini A, Dettling C, Thomas U, Hunziker P. Isolation and characterization of four bactericidal domains in the bovine β-lactoglobulin. *Biochim. Biophys. Acta* 2001; **1526**: 131–140.
20. Niidome T, Anzai S, Sonoda J, Tokunaga Y, Nakahara M, Hatakeyama T, Aoyagi H. Effect of amino acid substitution in amphiphilic α-helical peptides on peptide-phospholipid membrane interaction. *J. Peptide Sci.* 1999; **5**: 298–305.
21. Lee S, Park NG, Kato T, Aoyagi H, Kato T. Effect of proline in basic and α-helical amphipathic peptides on acidic liposomes and their antimicrobial activity. *Chem. Lett.* 1989; 599–602.
22. Mihara H, Kanmera T, Yoshida M, Lee S, Aoyagi H, Kato T, Izumiya N. Design and synthesis of

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amphiphilic basic peptides with antibacterial activity and their interaction with model membrane. *Bull. Chem. Soc. Jpn.* 1987; **60**: 697–706.
23. Krishnakumari V, Nagaraj R. Addition and omission analogs of the 13-residue antibacterial and hemolytic peptide PKLLKTFLSKWIG: structural preference, model membrane binding and biological activities. *J. Peptide Res.* 1999; **53**: 47–55.
24. Hong J, Oren Z, Shai Y. Structure and organization of hemolytic and nonhemolytic diasteromers of antimicrobial peptides in membranes. *Biochemistry* 1999; **38**: 16 963–16 973.
25. Ohmori N, Niidome T, Hatakeyama T, Mihara H, Aoyagi H. Interaction of α-helical peptides with phospholipids membrane: effects of chain length and hydrophobicity of peptides. *J. Peptide Res.* 1998; **51**: 103–109.
26. Wade D, Andreu D, Mitchell SA, Silveria AM, Boman A, Boman HG, Merrifield RB. Antibacterial peptides designed as analogs or hybrids of cecropins and melittin. *Int. J. Peptide Protein Res.* 1992; **40**: 429–436.
27. Rex S. A Pro → Ala substitution in melittin affects self-association, membrane binding and pore-formation kinetics due to changes in structural and electrostatic properties. *Biophys. Chem.* 2000; **85**: 209–228.
28. Dempsey CE. Quantitation of the effects of an internal proline residue on individual hydrogen bond stabilities in an alpha-helix: pH-dependent amide exchange in melittin and [Ala-14] melitin. *Biochemistry* 1992; **31**: 4705–4712.
29. Dempsey CE, Bazzo R, Harvay TS, Syperek I, Boheim G, Campbell ID. Contribution of proline-14 to the structure and actions of melittin. *FEBS Lett.* 1991; **281**: 240–244.
30. Jacob J, Duclohier H, Cafiso DS. The role of proline and glycine in determining the backbone flexibility of a channel-forming peptide. *Biophys. J.* 1999; **76**: 1367–1376.
31. Tieleman DP, Shrivastava IH, Ulmschneider MR, Sansom MSP. Proline-induced hinges in transmembrane helices: possible roles in ion channel gating. *Proteins Struct. Funct. Genet.* 2001; **44**: 63–72.
32. Kaduk C, Dathe M, Bienert M. Functional modifications of alamethicin ion channels by substitution of glutamine 7, glycine 11 and proline 14. *Biochim. Biophys. Acta* 1998; **1373**: 137–146.
33. Chia BCS, Corver JA, Mulhern TD, Bowie JH. Maculatin 1.1, an anti-microbial peptide from the Australian tree frog, *Litoria genimaculata*: solution structure and biological activity. *Eur. J. Biochem.* 2000; **267**: 1894–1908.
34. Suh JY, Lee YT, Park CB, Lee KH, Kim SC, Choi BS. Structural and functional implications of a proline residue in an antimicrobial peptide gaegurin. *Eur. J. Biochem.* 1999; **266**: 665–674.
35. Matsuzaki K, Yoneyama S, Miyajima K. Pore formation and translocation of melittin. *Biophys. J.* 1997; **73**: 831–838.
36. Eisenberg D. Three-dimensional structure of membrane and surface protein. *Annu. Rev. Biochem.* 1984; **53**: 595–623.

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