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Review

Action mechanism and structural requirements of the antimicrobial peptides, gaegurins

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ABSTRACT

Gaegurins (GGNs) are a family of cationic, α-helical, antimicrobial peptides that were isolated from a Korean frog, *Glandirana emeljanovi* (formerly classified as *Rana rugosa*) and represent one of the structurally well-characterized groups. Among six gaegurins, gaegurin 4 (renamed herein esculentin-2EM), gaegurin 5 (brevinin-1EMa), and gaegurin 6 (brevinin-1EMb) have been investigated comprehensively in terms of structure–activity relationships. In this paper, we first suggest renaming of gaegurins according to a recently raised rule of systematic nomenclature. Then, the current understanding of gaegurins is reviewed by summarizing their structure–activity relationships. In particular competing arguments on gaegurins are synthetically inspected. Finally their action mechanism and structural requirements will be discussed.

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1. Reflections and updates

The anuran skin has served as an exceedingly rich source of antimicrobial peptides [1–3]. Several hundreds of antimicrobial peptides with approximately 10–50 amino acids have been isolated from various species of frogs and many of them have been considered as a potential source of therapeutic agents [1–6]. Gaegurins (GGNs) are one of the early isolated groups of frog-skin antimicrobial peptides that manifest a broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, and protozoa, with little hemolytic activity [7–10]. Anticancer activities have been also

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reported for GGN5, GGN6, and their derivatives [9,10]. Accordingly, GGN4, GGN5, and GGN6 among six GGNs have been structurally well-characterized to be investigated in terms of therapeutic development. Prior to the review on their structural aspects, we first clarify the frog species and the peptide names, in agreement with the recent suggestion of systematic terminology, since the taxonomy of gaegurins was proposed to be revised in accordance with a new classification of frog species and a systematic rule of nomenclature [11].

1.1. Isolation and identification of gaegurins

Gaegurins, a group of six membrane-active antimicrobial peptides, were first isolated in 1994 from the skin homogenate and extradermal secretion of a Korean frog, *Rana rugosa* [7]. Subsequently in 1995, two complete cDNAs encoding GGN4 and GGN5 including their precursor polypeptides were isolated from a library constructed with the frog skin mRNAs [12]. It was also confirmed that gaegurins are extensively expressed in the skin tissue, but not in liver and muscle. Finally, the gene organization of GGN4 and its regulation of expression were elucidated in 2000 [13]. Despite this clear identification, gaegurins from the species *R. rugosa* in Korea have provided a questionable example, since a different group of antimicrobial peptides were isolated from the same species of frog. Independent study by different laboratory on the skin extracts of the same species *R. rugosa* inhabiting in Japan resulted in isolation of three peptides named rugosins, which were different from gaegurins [14]. Although a high sequence similarity can be observed between rugosins and gaegurins (Fig. 1; GGN2 for rugosin A, GGN1 for rugosin B, and GGN4 for rugosin C), the appreciable differences could not be expected from exactly the same species. Similar examples, where different peptide groups were isolated from nominally the same species of frog in different geographical areas, are available from *Hylarana güntheri* and *Rana esculenta* [11,15,16]. These confusing results alternatively suggest that taxonomic classification of frog species should be revised to reflect the considerable polymorphism. Fortunately, the discrepancy between gaegurins and rugosins is now resolved by recent advances in phylogenetic analysis of the family *Ranidae* (the order *Anura*; the class *Amphibia*). As *Ranidae* was one of the most diverse amphibian groups, the taxonomy of ranid species especially in the subfamily *Raniae* has been controversial and revised many times [17]. In particular, the genus *Rana*, which has been considered as the most diverse and worldwide group of anurans with approximately 250 species [18,19], is currently distributed to 16 different genera in *Ranidae* [20]. As noted by Conlon [11], according to the well-established taxonomy in the ASW database [20], the former species *R. rugosa* in Korea and Japan are reclassified as *Glandirana emeljanovi* and *Glandirana rugosa*, respectively. Thus, gaegurins and rugosins should be regarded as independent groups of antimicrobial peptides isolated from similar but different species of frogs.

1.2. Nomenclature of gaegurins

Molecular diversity of frog-skin antimicrobial peptides is often useful as taxonomic and phylogenetic markers to clarify evolutionary aspects [15,19,21,22]. Investigators of frog-skin antimicrobial peptides have named the newly identified peptides mainly referring to the name of frog species. However, this terminology was not valuable to reflect the evolutionary relationships. Despite the enormous reports of several hundreds of frog-skin antimicrobial peptides from ranid species, an established rule of nomenclature has not been shared and some names were arbitrary. As suggested recently by Conlon [11], the system of nomenclature used by Simmaco et al. [23,24] would be the best choice. In this naming, orthologs of an originally discovered parent peptide are characterized by the initial letter(s) of species, set in upper case, with paralogs being assigned letters set in lower case. Current 14 representatives for the parent peptides to designate peptide families were summarized by Conlon [11].

Gaegurins were the first isolation of frog-skin antimicrobial peptides in Korea and the authors named them referring to a Korean word, Gaegury, which means frog [7]. As shown in Fig. 1, the size and amino acid sequence of gaegurins exhibits a similarity to that of brevinins (brevinin-1 for GGNs 5 and 6 and brevinin-2 for GGNs 1 to 3) and esculentin-2 (for GGN4), which had been isolated earlier from the Japanese frog *Pelophylax porosus* (formerly classified as *Rana brevipoda porsa*) [26] and the European frog *Pelophylax lessonae*/*ridibundus* (formerly *R. esculenta*) [25], respectively. Therefore, according to the Conlon's scheme of taxonomy, the names of gaegurins are revisable as follows. GGN1 and GGN2, belonging to the brevinin-2 family, are revised to brevinin-2EMa and brevinin-2EMb, respectively, where the EM is derived from the frog species name (EMeljanovi). We suggest a new name brevinin-2EMb′ (or GGN2′) for GGN3, of which the sequence is almost the same as that of GGN2 (brevinin-2EMb) with only a single substitution at position 12. GGN4 has been roughly classified by Conlon [11] as a member of brevinin-2 family, together with GGNs 1–3. However, the longer peptide GGN4 is distinguished from GGNs 1 to 3 in the brevinin-2 family and shows a sequence similarity to esculentin-2 with the same number of amino acid residues (Fig. 1). Thus, GGN4 is assignable to the esculentin-2 family,

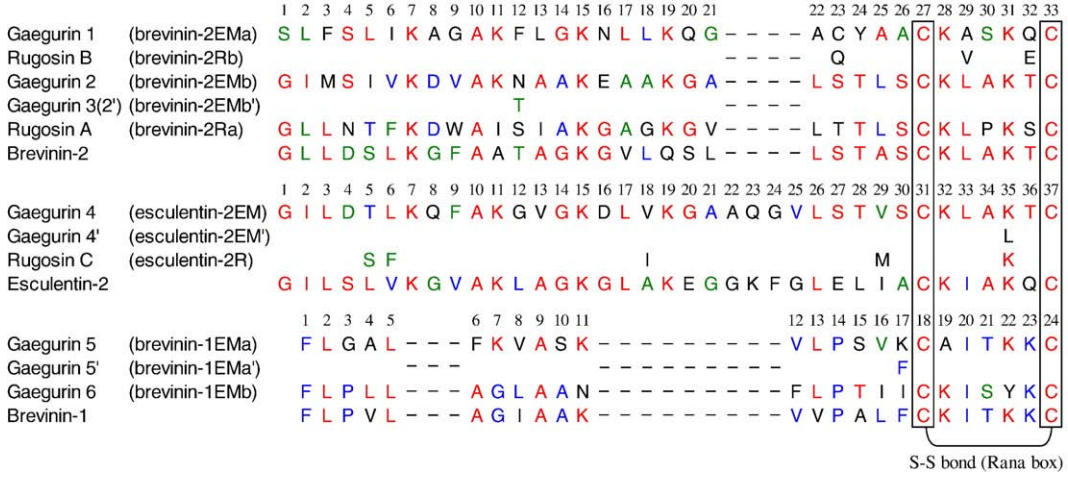

Fig. 1. Comparison of primary structures of gaegurins and their related peptides. New name of each peptide, suggested in this paper, is presented in parenthesis. Gaps (−) were introduced to maximize the sequence identities, which at aligned positions are indicated by the same colors. For clarity, the sequences of rugosin B, gaegurin 3, gaegurin 4′, rugosin C, and gaegurin 5′ are represented only at the positions with different amino acids from the very upper line sequences.

rather than to the brevinin-2 family, and can be replaced by esculentin-2EM. Finally, GGN5 and GGN6 in the brevinin-1 family can be renamed as brevinin-1EMa and brevinin-1EMb, respectively. Similarly, rugosins from *Glendirana rugosa* are revisable as brevinin-2Ra, brevinin-2Rb, and esculentin-2R, as indicated in Fig. 1.

In addition to gaegurins, some other peptide names from Korean fogs are now revisable. The peptides named esculentin-1c and brevinin-1Ed from the Korean *R. esculenta* [16] can be renamed as esculentin-1N and brevinin-1N, respectively, since the frog species is now assignable to *Pelophylax nigromaculatus* according to the newly updated taxonomy in the ASW database [20]. A brevinin-2 family peptide, nigrosin-1 from the Korean *Rana nigromaculata* [26], which can be now reclassified as *Pelophylax chosenica*, is desirable to be renamed as brevinin-2CH. Then, the peptide nigrosin-2 from the same species can be called just nigrosin. Since nigrosin has a unique primary structure, it should be used as a representative term to designate an independent peptide family, as also supported by Conlon [11].

1.3. Amino acid sequences of gaegurins

Amino acid sequences of the six gaegurins were originally revealed by peptide sequencing [7]. Among them, GGN4 and GGN5 were additionally supported by cDNA isolations [12]. However, the peptide sequence derived from cDNA indicated Lys 35 in GGN4 and Phe 17 in GGN5 instead of the formerly reported Leu and Lys, respectively. Then, the authors corrected the original sequences of GGN4 and GGN5, through an erratum report [Erratum of 7]. The structural research on GGN4 [27] has been performed on the latter sequence, since it was initiated using the cDNA clones. In contrast, the structure of GGN5 was investigated with a synthetic peptide of the former sequence [28], without recognition of the erratum report. However, since the relevant activities were checked, the determined structure would be still valid to interpret structure–activity relationships of GGN5. Moreover, it is not reasonable to conclude depending on the isolated cDNA that the original sequence was misread. In the case of brevinins and esculentins from the former *R. esculenta*, the peptide sequences deduced by cDNA cloning also showed slight differences from those reported originally from the skin secretion [23,24]. This result promoted more detailed examination of the skin secretion, and finally it was revealed that several variants with one or a few amino acid substitutions exist in a single family. GGNs also support this notion since GGN3 showed a single variation from GGN2 (Fig. 1). Thus, we suggest that all the GGN sequences shown in Fig. 1 are relevant to naturally occurring peptides. Exceptionally, we find that the GGN6 sequence has been mis-introduced in some literatures [4,26,28,29] by showing Phe instead of Lys 19. To revise the peptide names as in Fig. 1, we assigned GGN4 to the cDNA-derived sequence and GGN5 to the original sequence, since those sequences were employed for structure-functional investigations [9,27–32]. Then the other synonymous sequences, which have not been used for any other studies, were designated as GGN4′ (esculentin-2EM′) and GGN5′ (brevinin-1EMa′), respectively.

2. Structure–activity relationships of gaegurins

Molecular diversity of antimicrobial peptides is so great that it is very difficult to definitely categorize them in terms of structure. Just broadly, antimicrobial peptides are often classified into 3 structural classes [5,33,34]: the linear α-helical peptides, the peptides with several disulfide bonds stabilizing β-sheet or α/β structure, and the peptides with unusual predominance of certain amino acid(s) or unusual structure. Gaegurins belong to the first class, of which membrane permeation results in membrane disruption via a 'barrel-stave' or 'toroidal' pore formation or a 'carpet-like' disintegration of membrane [1,4,35–37]. Restricted to anuran-skin antimicrobial peptides, the following classification is also available [1]: linear amphipathic α-helical peptides, amphipathic α-helical peptides with a C-terminal disulfide bridge, and the other unique peptides with relatively shorter length or distinct disulfide bond. Gaegurins represent a group of cationic, amphipathic, α-helical, antimicrobial peptides with a C-terminal disulfide bond. The positive charges abundant in this group of peptides are important to generate selectivity, by interacting discriminatively with bacterial membranes that are heavily populated by lipids with negatively charged phospholipid headgroups and eukaryotic membranes, of which surface is principally zwitterionic with no net charge [5,33–40]. The amphipathic characteristic of the helix is crucial for membrane interaction and available by positioning hydrophobic amino acids on one side and hydrophilic residues on the other side from the helical axis (Fig. 2,Fig. 3,Fig. 4). The helical structure is often induced upon interaction with membranes, while the peptides are poorly structured in aqueous solution [5,38–40]. To induce that functional conformation relevant to peptide activity, experimental model systems mimicking membrane environments are often used for structure determination [37,41]. For example, organic co-solvents such as trifluoroethanol (TFE) provide a hydrophobic environment as the core of membrane, and detergent micelles such as sodium dodecylsulfate (SDS) and dodecylphosphocholine (DPC) micelles simply mimic the membrane structure. As a most advanced model membrane, lipid vesicles with various compositions are applied. The three-dimensional structures of gaegurins 4, 5, 6, and their analogues in membrane-mimetic environments are presently available, solved by NMR groups in Korea, and provide an insight into their structure–activity relationships.

2.1. Insight into the Rana box, the C-terminal cyclic heptapeptide with a conserved disulfide bond

All of the six gaegurins possess two strictly conserved cysteines that form a disulfide bond (Fig. 1). The cyclic heptapeptide region by this disulfide bond at the C-terminus is called 'Rana box', since it was frequently observed in majority of ranid antimicrobial peptides. Among the 15 peptide families from *Ranidae* (14 families categorized by Conlon [11] plus herein tigerinins [42]), only temporins are devoid of the C-terminal cyclization. Some modifications of the Rana box are observed in japonicin-2, palustrin-2, ranacyclin, ranatuerin-2, and tigerinin families, where the C-terminal position and/or the peptide size of cyclization are different. The Rana box region adopts a helical loop-like fold stably constrained by the disulfide bridge (Fig. 4) [26–30,43]. Functional importance of this conserved moiety can be simply inferred from the fact that C-terminal truncations of the peptides often diminish their antimicrobial activity. For example, an ranalexin analogue with a single amino acid deletion of the last cysteine and a GGN4 analogue where the C-terminal nine residues were truncated had markedly reduced antimicrobial activity [44,45]. GGN6 becomes insoluble upon truncation of the Rana box [10]. However, the exact role of the Rana box still remains ambiguous and controversial and it is arguable whether the conserved disulfide bond is always a requirement for activity.

2.1.1. Reduction of the disulfide bond

Researches on the structural and functional role of the disulfide bridge have employed either a reduced form with free cysteines [27,28,43,46] or a linear mutant where the cysteines were replaced by serines or chemically modified to be unable to form disulfide bond [28,46–51]. The reduced form was confirmed by monitoring the change of molecular mass, NMR spectra, and/or susceptibility to carboxypeptidase Y that cleaves polypeptides from the C-terminus [27]. The reduced GGN4 and GGN5, compared with the native (oxidized) ones, did not show any significant difference in activity [27,28]. Even the three-dimensional structures in membrane-mimetic environments were very similar to those of the native (oxidized)

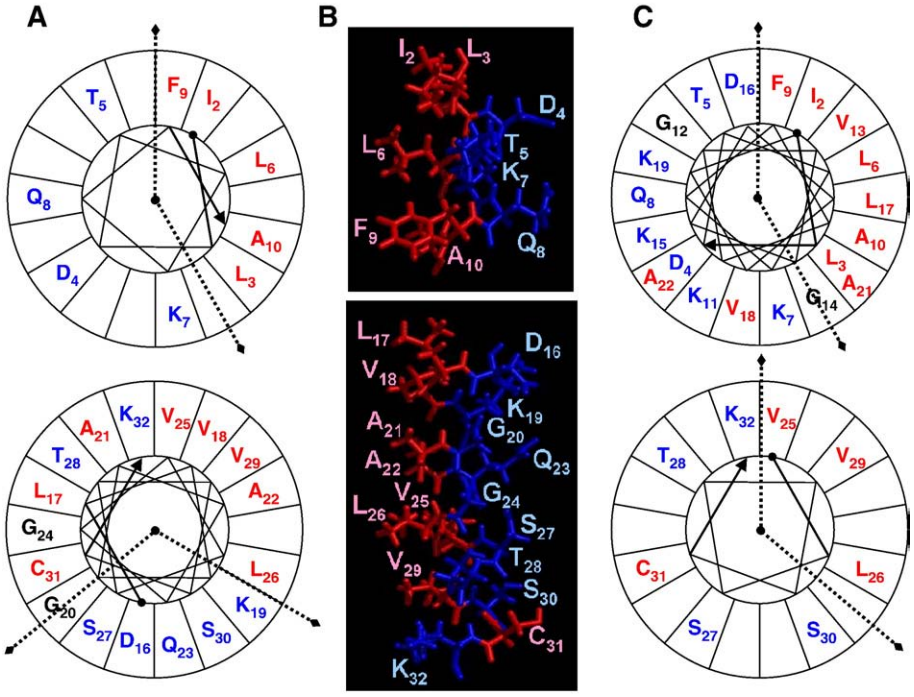

Fig. 2. Amphipathic characteristics of the N-terminal (top panels) and the C-terminal (bottom panels) helix of GGN4 in 50% TFE/water (A, B) and in 80% methanol/water (C), illustrated as helical wheel diagrams (A, C) and a three-dimensional structure (B). Hydrophobic residues are colored red and hydrophilic residues blue. Glycines are regarded as a neutral amino acid (A, C) or a hydrophilic amino acid (B). The interface between the hydrophobic and the hydrophilic sides is indicated by dotted arrows (A, C).

forms, with their helical length shortened just by one or two residues at the C-terminus. Despite the loss of disulfide bond, the loop-like fold at the Rana box region was preserved, although it was rather loosely defined in the reduced forms. Even in the reduced forms, the central residues of the Rana box showed a slow exchange of the backbone amide protons, probably due to hydrogen bonds [27,28]. Similar observations were obtained from the ranalexin (Rnx) and brevinin-1E, where the reduction of the disulfide bond resulted in a just tolerable decrease of activity [43,46,47]. The helical contents of the reduced and oxidized brevinin-1E amide, deduced from CD spectra in TFE and various concentrations of SDS, were comparable to each other [46,47]. In the three-dimensional structure of the reduced Rnx, determined in detergent micelles, the helix was just two residues shorter at the C-terminus, and the C-terminal loop structure was roughly close to that observed in native (oxidized) Rnx [43]. All these consistent results of GGN4, GGN5, Rnx and brevinin-1E suggest that the disulfide bond does not play a major role in structure and activity. Exceptionally, it was noteworthy that the deletion of the C-terminal cysteine of Rnx remarkably impaired its activity [44]. Thus, at least in Rnx, the cysteine residue at the C-terminal end is crucial for activity, regardless of disulfide bond formation.

Similar approach to reduced GGN6 showed a contrary result [48]. The reduced GGN6 amide almost completely lost the antimicrobial activity while the oxidized form was fully active. Conformational behaviors were also different between the reduced and oxidized forms. The oxidized form maintained approximately 70% (estimated by CD spectroscopy) α-helix contents in the presence of 1.25 to 25 mM SDS. Although the helical content of the reduced form in 25 mM SDS solution was comparable to that of the oxidized form, it was appreciably decreased at 2.5 mM SDS, which is below the SDS critical micelle concentration (8.1 mM; [52]). In addition, at lower concentration (1.25 mM) of SDS, the reduced form was not soluble and precipitated. Similarly, the truncation of the Rana box in GGN6 made the peptide insoluble in water [10]. Since the majority of hydrophilic amino acids in GGN6 are located within the Rana box (Fig. 1), the insolubility of the N-terminal fragment lacking the Rana box could be expected from its strong hydrophobicity with no charged residues. In contrast, the precipitation of the reduced GGN6 suggests

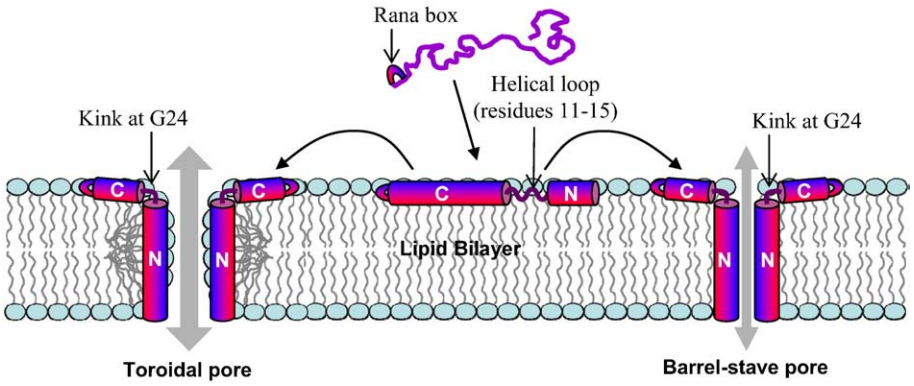

Fig. 3. Model for membrane permeation of GGN4, suggested in this review. The N-terminal and the C-terminal amphipathic helices are represented by cylinders, with blue color for hydrophilic and red for hydrophobic face. The ion channels formed by helical bundle of GGN4 are indicated by up-down arrows. For clarity, only two monomer units of the GGN4 aggregates in the ion channels are displayed.

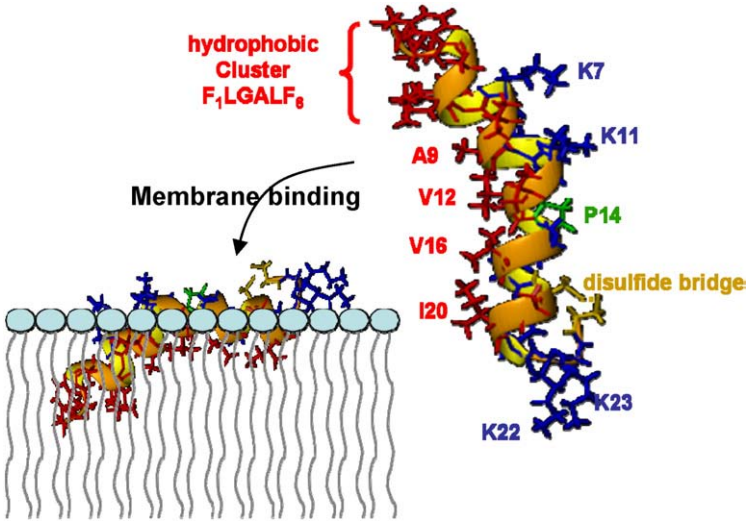

Fig. 4. NMR-derived solution structure of GGN5 in SDS micelles and its membrane-binding model. Backbone structure is displayed as a ribbon model with side chains colored red, blue, green and yellow for hydrophobic, hydrophilic, proline and cysteine residues, respectively.

that the peptide possess a quite lower propensity to adopt a proper amphipathic helical structure and/or a higher tendency to form unfavorable aggregates.

The decreased helical propensity in reduced form has been also observed from Rnx and brevinin-1E just in water [43,46,47]. Although poorly structured, small amount of helical propensity in the 12-16 segment could be evidenced by NMR spectroscopy, for the native (oxidized) Rnx but not for its reduced form [43]. The far-UV CD spectrum of the native (oxidized) brevinin-1E amide also indicated a detectable amount of helical content in water, but the spectrum of reduced form did not [46,47]. These observations are distinct from the result of GGN6, in that the decreased helical propensity of the reduced Rnx and brevinin-1E in water did not have a critical effect on their activity, as described above. Nevertheless, another approach to the reduced brevinin-1E is in support of the result from reduced GGN6. The N-terminal two- or five-residue truncation of the hemolytic peptide brevinin-1E results in a moderately impaired antimicrobial activity with a remarkably decreased hemolytic activity [46]. The reduction of the disulfide bond in this fragment still has no significant effect on the antimicrobial activity and helical adoption in SDS micelles. However, at 1.25 mM SDS where the reduced GGN6 precipitated, the CD spectrum of the reduced brevinin-1E fragment appeared in a totally different pattern, indicative of a β-sheet structure and/or aggregations. Finally, it has been revealed that the reduction of the fragment further decreased hemolytic activity without significant effect on antimicrobial activity.

In summary, the comparisons between oxidized and reduced forms of gaegurins and their related peptides showed that the conserved disulfide bond can stabilize the helical propensity correlated with activity but would be differently applied to each peptide.

2.1.2. Substitution of cysteines

The results with linear mutants lacking the disulfide bridge are also contradictory to one another. The linear mutant of GGN5, where the two cysteines were substituted with serines, showed a remarkable decrease of activity and helical content [28]. This result is not consistent with that observed on the reduced forms of GGN4 and GGN5 but in agreement with the result of the reduced GGN6 amide (refer to the Section 2.1.1.). In the high-resolution structure, the C-terminus of the linear GGN5 mutant frayed to be flexible and disordered even in SDS micelles, inconsistent with the loop-like fold shown in both the oxidized and reduced forms of native GGN5. In contrast, the linear (cysteines to serines) mutant of GGN6 in TFE/water showed a stable α-helix elongated up to the C-terminus, despite the C-terminal substitutions [49]. Accordingly, the activity of the linear GGN6 was comparable to that of native GGN6 [48–50]. Similar behavior was observed from a linear analogue of brevinin-1E possessing acetamidomethylated (thus unable to form a disulfide bridge) cysteines. This analogue also showed the activity and the helical propensity just marginally decreased from those of native one [46,47]. Another example with a linear mutant of esculentin-C, an active variant of esculentin-1, showed a similar result as GGN6, but suggests an alternative role of the disulfide bond for exerting activity [51]. The substitution of cysteines with serines in the esculentin-C did not significantly affect the antimicrobial activity, evaluated as lethal concentration. However, the bacterial killing kinetics, monitored by time-killing courses, was different: the effect of the linear mutant was slower than that of the cyclic molecule. An interesting approach to the Rana box has been also constructed on brevinin-1E, by transposing the C-terminal Rana box to a central location [47]. The native brevinin-1 has potent hemolytic activity as well as antimicrobial activity, unlike gaegurins that possess little hemolytic activity. However, the brevinin-1E analogue with the transposed Rana box (namely BA) showed a favorable selectivity: i.e. the antimicrobial activity was retained or slightly increased but the hemolytic activity was 10-fold lowered. Then, the reduced form of the analogue (namely BAL) showed no detectable hemolytic activity. This result could be interpreted from the viewpoint of electrostatic charge interaction with membrane surface. The N-terminal half of brevinin-1E is mostly hydrophobic while the three lysines are localized in the C-terminal region, in particular two lysines being constrained in the Rana box. Thus the transposition of the Rana box to central position would confer a favorable distribution of positive charges, thus preferentially stimulating the interaction with negatively charged membranes of bacteria. Then, the transposed Rana box without disulfide bond would further favor effective interaction with negatively charged bacterial membranes but not with zwitterionic eukaryotic membranes. Indeed it was confirmed by CD spectroscopy that brevinin-1E and the analogues show a different and dynamic conformational behavior in various lipid vesicles with different surface charge [47].

2.1.3. Concluding remarks: structural and functional implications

Based on this review, the antimicrobial activities of gaegurins and their related peptides correlated with their propensity to stably adopt the helical structure favorable for membrane permeation. For this, the Rana box seems to play a role of C-terminal capping to stabilize the helical structure, thus complementing a stable activity.

The specific role of the Rana box in GGN4 will be further discussed in the following sections. The C-terminal disulfide bond can also provide a resistance to proteases such as carboxypeptidase Y. However, sometimes the disulfide bond appears unnecessary for activity. Thus, it seems to depend on the primary structure of each peptide how much the conserved disulfide bond is critical for the activity. In some cases, the conserved cysteine residues are crucial for activity, irrespective of disulfide bond formation. To clearly rationalize the necessity or a common contribution of the conserved disulfide bond, more systematic and comparative experiments would be necessary for a group of related peptides including GGNs 1–3. However, there might be no coherent role of the moiety, thereby just indicating a molecular diversity and individual specificity obtained during evolution.

2.2. Action mechanism of gaegurin 4 (esculentin-2EM)

GGN4, a 37-residue antimicrobial peptide, is the longest one of gaegurins and most well characterized in structure and functional mode. However, there are currently some conflicting arguments on the structure–activity relationships of GGN4. A synthetic insight would provide a clue to interpret its action mechanism.

2.2.1. Pore-forming activity of GGN4

The membrane-lytic activity of antimicrobial peptides is often directly measured by a dye leakage experiment in model membrane systems, which represents one of the recent technical improvements to gain a deeper insight about the lytic action [53]. Although the membrane-lytic activity of GGNs has not yet been characterized by that experiment, GGN4 has been suggested to function via a pore-forming process since the ionophoric properties were evidenced by electrophysiological approaches [54–56]. In brief, GGN4 formed voltage-dependent and cation-selective pores in planar lipid bilayers. The pore-forming activity was dependent on the peptide concentration [54] and recently a helix-induced oligomeric (maximally 10-mer) transition of GGN4 was observed in a 15% hexafluoro-2-propanol (HFIP)/water solution [55]. Details are as follows.

Originally, the voltage-gated ion channels induced by GGN4 were inferred in planar lipid bilayers under voltage clamp, by checking the membrane conductance increasing upon addition of GGN4 [54]. When the membrane potential was defined as that of the peptide-added compartment with respect to that of the other compartment (i.e., the current flow from the peptide-added compartment to the other compartment is defined as 'outward current'), the gate-like activity was voltage-dependent, thereby increasing at more negative voltages but rarely at positive voltages. The activity was also rarely observed at low (~0.03 μg/ml) concentration of GGN4 applied. As the peptide concentration increased, the bilayers were more frequently broken with larger change of conductance. The pore-forming activity in acidic lipid bilayers was similar to or slightly higher than that in neutral bilayers [54,56]. Considering the positively charged characteristic of GGN4, it was anomalous that the ion channels formed by GGN4 were selective for cations such as K^+ and indeed contradictory to the anion selectivity of other known basic peptides [54]. Even lager cations such as TEA^+ and $NMDG^+$, of which geometrical mean diameters are 6.6 and 7.3 Å, respectively, were also transportable through the channels, although their permeability was lower than that of K^+ [55]. To allow the $NMDG^+$ to pass, the diameter of the GGN4-induced pores should exceed 7.3 Å that requires at least pentameric barrel-stave assembling of GGN4 [55]. Alternatively, as discussed by Eun et al. [55], it is likely that GGN4 forms various oligomers in real membranes, thereby providing heterogeneous pores with different sizes. In this case, the toroidal pore that has a variety of sizes and lifetimes [4,35,36] is more suggestible for the GGN4-induced ion channel, rather than a barrel-stave pore, which is expected to have a consistent channel size [4,35,36]. The heterogeneity of the GGN4-induced pores had been reflected from the fact that GGN4 induced widely heterogeneous conductance in the planar lipid bilayers [54].

The ionophoric activity of GGN4 was also evaluated in bacterial cells and human erythrocytes, by measuring K^+ efflux [56]. The K^+ efflux in Gram-positive bacteria (*M. luteus*) showed higher potency and efficacy and more rapid kinetics than that in Gram-negative bacteria (*E. coli*). In contrast, all those parameters indicated a poor activity in human red blood cells. These results well correlate with the selective toxicity of GGN4. GGN4 exhibits more potent antimicrobial activity against Gram-positive than Gram-negative bacteria, but little hemolytic activity [7,27].

2.2.2. Role of Rana box in pore formation

The ionophoric activity of GGN4 has been also examined for its reduced form and C-terminally truncated analogues, to deduce the role of the C-terminal Rana box (residues 31–37). The reduction of the C-terminal disulfide bridge between Cys 31 and Cys 37 did not have significant effect on the pore-forming activity of GGN4 [56]. This is consistent with the previous result that showed no significant alteration of structure and antimicrobial activity, upon reduction of the disulfide bond [27]. In contrast, truncation of the Rana box resulted in a remarkable decrease of ionophoric activity. The N-terminal 30-residue fragment lacking the Rana box was quite less potent in inducing membrane conductance in planar lipid bilayers and in inducing K^+ efflux from both Gram-positive and Gram-negative bacteria [56]. Quantitatively, the ionophoric activity of the Rana box-truncated fragment was less than 1/10 of the intact GGN4 activity, or even negligible. However, the properties of the ion channels were qualitatively preserved; i.e. the channels were selective to cations and more potent in Gram-positive bacteria. Unfortunately, the precise antimicrobial activity of the 30-residue fragment has not been evaluated but a substantial decrease of antimicrobial activity was confirmed from another fragment with N-terminal 28 residues of GGN4 [45]. Then, further truncation from the C-terminus impaired more severely the antimicrobial activity. The N-terminal 23- and 22-residue fragments of GGN4 showed no significant activity [32,45]. The absence of ionophoric activity in planar lipid bilayers was also confirmed for the 23-residue fragment [54]. Taken all together, the C-terminal disulfide bond of GGN4 is not a requirement for its activity but the C-terminal parts including the Rana box region is crucial for activity, probably by complementing the membrane affinity rather than by participating in the transmembrane pore structure.

2.2.3. Structural insight

GGN4 is mostly unstructured in aqueous solution, as simply evidenced by its far-UV CD spectrum that is characterized by a single, strong negative band near 200 nm [27]. Only a nascent turn near the Rana box could be supported in water by a detailed NMR study [29]. In the presence of organic co-solvents (TFE or methanol) or detergent (SDS or DPC) micelles, the peptide is folded into a stable α-helical conformation [27,29,30], which would represent its functional structure. However, precise examinations of the three dimensional structure by NMR have shown conflicting results dependent on the solvent. The structures determined earlier in 50% TFE/water mixture [27] and in SDS micelles [30] showed a helix–loop–helix conformation with two α-helices extending from residues 2 to 10 and from residues 16 to 32, linked by a flexible hinge spanning between the residues 11 and 15. The loop region (residues 11–15) assumed a helix-like conformation but was flexible, thereby allowing an independent movement of both helices without contact, at an angle of approximately 60°–120° to each other [27,30]. In contrast, the recent structure in 80% methanol/water mixture indicated a helix–kink–helix topology with a long helix encompassing residues 2–23 and the other consisting of residues 25–34, intervened by a helix break at Gly 24 [29]. The bending angle between helices connected by the flexible

kink was predicted as 60°–150°. In any case, each helical segment exhibits a completely or highly amphipathic characteristic as can be seen in Fig. 2. However, it is not clear which structure is relevant to the real functional state in biological membranes, which is discussed in next section.

The former structure of GGN4 in TFE/water and in SDS micelles could be validly determined since the peptide was prepared as a recombinant protein, which was readily applied to isotope labeling for heteronuclear NMR as well as homonuclear NMR [27,30]. The arrangement of helices was additionally supported by heteronuclear NOE experiments in SDS micells [30]. The result suggested that the interhelical loop region (residues 11–15) determined is slightly more flexible than the helices. Additional structure of the N-terminal 23-residue fragment of GGN4 in 50% TFE/water also supported the presence of the interhelical loop. In this structure, the N-terminal helix was preserved with similar length (residues 2–11) but the other parts were predominantly frayed, probably due to the break of the peptide bond at position 23 [32]. Then, a tryptophanyl substitution at position 16 of the fragment stabilized the helical structure up to residue 18 in TFE/water and to 20 in SDS micelles. Since the position 16 in the intact GGN4 is at the border between the loop region (residues 11–15) and the C-terminal helix (residues 16–32), it was reasonable to infer that the tryptophan stabilized the potential helical propensity of the previous loop region as well as the C-terminal helix destabilized by truncation [32].

The latter structure of GGN4 in methanol/water was determined with a synthetic peptide but it could be also unambiguously determined by employing very careful and massive NMR experiments [29]. Additionally, the arrangement of helices broken at Gly 24 could be supported by sequence comparison of related peptides. Inspection of amino acid sequences found many potential or observed helix break occurring near a position matchable to Gly 24. The structure in methanol/water was also useful to draw a very plausible model for the pore-forming mechanism of GGN4. The suggested model from this structure depicts that the long N-terminal amphipathic helix inserts perpendicularly into the membrane and assembles into an oligomeric bundle that forms an ionophore. This seems probable since the helical length with 22 residues is able to span the membrane and the amphipathic properties are generally requisite for helical bundle to form ion pore in membranes [57]. The flexible kink at Gly 24, in this model, allows the C-terminal helix to lie on the membrane surface. This is also likely in that the C-terminal Rana-box contains two lysines of which positive charges would favor electrostatic interaction with the negatively charged surface of bacterial membrane. In addition, as noted above (refer to the Section 2.2.2.), it has been revealed that the Rana box in GGN4 is not involved in the pore structure spanning membranes but effects on the potency; i.e. the Rana box-truncated fragment of GGN4 formed the ionophore at higher concentration [56].

2.2.4. Model for membrane permeation suggested

Strictly speaking, GGN4 forms ion pores rather than channels, which tend to be more selective. Presently it is not clear which one of the two different conformation of GGN4 is relevant to the real functional state in biological membranes. However, an alternative possibility that both of the two structures would be involved in action mechanism cannot be excluded. The model encompassing both the two structures is suggestible as in Fig. 3. Generally it is accepted that the primary binding of the pore-forming peptides to membrane occurs on the membrane surface with inducing helical structure from the random structure in aqueous solution. The amphipathic helix is requisite to stabilize this initial binding, where the hydrophilic face interact with the lipid head groups and hydrophobic face contacts to the inner parts of lipids with acyl chains. Finally the ionophore is suggested to be formed transiently by oligomerization of the peptides in a concentration-dependent manner. Based on this general mechanism of pore-forming peptides [34–41], the former helix–loop–helix structure of GGN4 would be responsible for its primary binding while the latter helix–kink–helix structure is assignable to the ion pore formation as a barrel-stave or a toroidal pore (Fig. 3). The heteronuclear NOE results suggested that the micelle-bound helix–loop–helix peptide had some mobility of the helices, which would be related to a membrane permeation process. In contrast, the helix–kink–helix conformation possesses very suitable properties for ionophore structure as discussed above (refer to the Section 2.2.3.).

A basic premise of the model in Fig. 3 is an additional conformational change from the helix–loop–helix structure to the helix–kink–helix structure during oligomerization and/or spontaneous insertion into membrane. As noted herein, the interhelical loop region of the helix–loop–helix was flexible but adopted a helix-like conformation [27,30] and showed a potential helicity that can be readily stabilized [32]. In addition, heteronuclear NOE result of GGN4 in SDS micelles indicated that the residue Gly 24, as well as the loop region, is rather flexible. Thus, the helix–loop–helix conformation observed in TFE/water and in SDS micelles possesses a high potential to be readily converted into the helix–kink–helix structure observed in methanol/water.

Despite the plausible reasoning, the suggested model might be criticized by the recent observation of GGN4 oligomerization. The analytical ultracentrifugation of GGN4, combined with CD and fluorescence spectroscopy, indicated solely monomeric state in methanol/water. In contrast, in the presence of a fluorinated alcohol HFIP (hexafluoro-2-propanol), higher order aggregates were detected [55]. HFIP, like TFE, is a strong enhancer of α-helix formation and it has been additionally suggested to be able to form micelle-like aggregates with water. Thus, this result is in opposition to the suggested model in Fig. 3, where the helix–kink–helix structure in methanol/water was assigned to the oligomeric state and the helix–loop–helix structure, solved in TFE/water and in SDS micelles, was represented as a monomeric form. However, previously in TFE/water and in SDS micelles [27,30], no detectable oligomerization of GGN4 had been indicated by CD and NMR parameters such as the ellipticity ratio at 222 nm to 208 nm [55,58] and NMR line broadening. Thus the two types of GGN4 structures reflect its dynamic conformational preferences whether it associates or not. The extent of GGN4 oligomerization in real membranes has been suggested to be various from pentamers to decamers [55]. From this point of view, the suggested conformational change in Fig. 3 from helix–loop–helix to helix–kink–helix would not be responsible for oligomerization but attributable to the transient membrane insertion. It has not been revealed whether the GGN4-induced ion pore is formed as a barrel-stave pore or a toroidal hole. The model depicted in Fig. 3 is effective for both the mechanisms. However, if the toroidal pore is practical, both the two structures of GGN4 might be favorable for its formation and adopt no particular orientation [1,4,35–37]. To clarify the correct mechanism, complementary experiments would be necessary in lipid bilayers to detect both the two helical conformations and to define their orientation.

2.3. Structural implications of gaegurins 5 and 6 (brevinin-1EMa and 1EMb)

GGN5 and GGN6, the 23-residue peptides, are the shortest ones of gaegurins and commonly belong to the brevinin-1-like family (Fig. 1). They undergo conformational changes as GGN4 does, from a disordered structure in aqueous solution to a helical structure in membranous environments. However, GGN5 and GGN6 are different from the longer peptide GGN4, in that they possess a single α-helix while GGN4 consists of two α-helices. As expected from their sequence similarity, the overall structures of GGN5 and GGN6 are also similar to each other. However, the same modifications of the Rana box had quite different effects between GGN5 and GGN6, as summarized above (refer to the Section 2.1.). In addition, specific roles

of another key moiety, proline kink appear to be different between the two peptides, although they commonly possess that moiety. A detailed and comparative inspection of the structural requirements is provided as follows.

2.3.1. Structural characteristics

High-resolution structures have been obtained for synthetic peptides corresponding to the native GGN5 and a linear (cysteines to serines) mutant of GGN6 [28,49,50]. Since the GGN6 mutant (^{CS}GGN6) was as active as native GGN6, it could represent the critical features in structure of native GGN6 [48–50]. The structures have been solved in SDS micelles for GGN5 and its analogues [28] and in TFE/water mixture for ^{CS}GGN6 and its analogue [49,50]. The overall structures of GGN5 and ^{CS}GGN6 are very similar with a single amphipathic helix encompassing residues 3–20 and 4–24, respectively. The N-terminal end position (position 3) of the helix is occupied by glycine in GGN5 and proline in GGN6, which amino acids in polypeptides are often appear at the end of a helix to function as a helix breaker or a helix capping. Generally in NMR structures, it is hard to definitely define the boundary of a helix and thus it is also arbitrary whether the Gly 3 and Pro 3 in the GGN5 and ^{CS}GGN6 should be included in the helix element or not. Considering together the different configuration at the C-terminus of ^{CS}GGN6, it can be regarded that GGN5 and GGN6 assume a nearly identical conformation.

The most unique feature of the GGN5 and GGN6 structure is a stable kink of the helix at the conserved Pro 14 (Fig. 4). Normally, proline is not favorable for helix formation because it cannot participate in the sequential hydrogen bonding network occurring in a helix, due to the absence of amide proton. However, the flanking residues of the Pro 14 was sequentially linked in a continuing helix [28,48,49]. The resultant was not a helix breaking at Pro 14 but only the helical kink, through which the helix is persistent. Thus, the kinked helix of GGN5 and GGN6 should be discriminated from the helix–kink–helix of GGN4 in methanol/water. Amphipathic peptides often adopt a curved α-helix [28,48,49,59]. The helices in GGN5 and ^{CS}GGN6 also seem to be intrinsically a little (about 10°) curved. Then, the helix becomes more bent by the Pro 14 that adopted the *trans* peptide bond. The bending angle by the proline kink has been estimated as about 25° [28], which is similar to that noted in many other proline-containing helices [60]. The kinked helix exhibits a typical amphipathic characteristic, where hydrophilic residues occupy the convex face and hydrophobic residues converge into the concave side (Fig. 4). In additional to the amphipathic properties, a convergence of hydrophobic amino acids at the N-terminus is also characteristic (Figs. 1 and 4).

2.3.2. Membrane binding mode and structural requirements

The membrane-bound orientation of GGN5 has been investigated by spin label experiments in SDS micelles [28]. Mn^{2+}, 5-doxyl stearate, and 12-doxyl stearate were employed as the paramagnetic probes monitoring the peptide location on the surface, beneath the surface, and in the core of the micelles, respectively. The hydrophilic residues in the convex of the helix and particularly at the C-terminal region were located on the surface of the micelles, while the hydrophobic residues in the concave and in particular at the N-terminus were predicted under the surface of the micelles. From the results, Park et al. [28] have proposed a plausible model for the initial membrane-binding mode of GGN5, as shown in Fig. 4. Basically this orientation facilitates and stabilizes both the charge interaction of cationic amino acids with the anionic membrane surface and the hydrophobic interaction of hydrophobic amino acids with the inner parts of membrane. Thus, as suggested generally, the inherent helicity and its amphipathicity would be a primary factor influencing the interaction of GGN5 and GGN6 with membranes. Then, another characteristic feature of the binding mode in Fig. 4 is provided by the proline kink. The helical kink favors a diagonal binding of the peptides to membrane, thereby anchoring the N-terminal hydrophobic cluster more deeply into the core of membrane and exposing the cationic amino acids in the Rana box onto the surface (Fig. 4). Particularly in GGN6, the hydrophilic residues and especially all lysines are localized in the C-terminal Rana box (Fig. 1). Thus the helical kink of GGN6 would be more efficient to separately arrange the hydrophobic N-terminus and cationic C-terminus, at the amphipathic interface of the lipid bilayers.

The functional implications of the Rana box were inspected above in the Section 2.1. The functional importance of the N-terminal hydrophobic cluster is verified by the N-terminally truncated analogues. Deletion of the 1–3 or 2–4 segment in GGN6 completely abolished its antimicrobial and anticancer activities [10]. The N-terminal 2–14, 3–13, 3–15, 4–16, and 5–17 fragments of GGN5 rarely exhibited antimicrobial activities, while the 1–13 fragment was highly potent [31]. In other related peptides such as brevinin-1E and Rnx, the N-terminal truncations often resulted in a striking decrease of activity [44,46].

In order to assess the functional role of the proline kink, the alanine derivatives (P14A) of GGN5 and ^{CS}GGN6 have been examined in structure and activity [28,49]. Structural effect of the P14A substitution was similar between GGN5 and ^{CS}GGN6. Although the P14A analogues still adopted a curved helix, the outstanding kink at position 14 disappeared and the substituted alanine was involved in the intra-helical hydrogen bonding network. Finally, the helical propensity (or the extent of helicity) and stability (or rigidity) were increased in the P14A analogues. Nonetheless, the effect of the substitution on activity was quite different between the two peptides. The P14A substitution in the ^{CS}GGN6 showed a substantial decrease of antimicrobial activity with still little hemolytic activity [49]. Similar observation has been obtained from the maculatin 1.1, a 21-residue antimicrobial peptide from Australian tree frogs, where the alanine substitution at Pro 15 led to loss of activity [61]. In contrast, the P14A substitution in GGN5 had little effect on the antimicrobial activity but greatly increased the hemolytic activity [28]. Thus, the helical kink in GGN5 and GGN6 seems to be important for both the activity and selectivity of the peptides, but the extent to which the kink affect would be different depending on amino acid composition. In particular, based on the following evidences, the proline kink appears to drive electrostatic interaction between the peptides and membranes, which would be important for its selectivity to bacterial cells. First, the P14A ^{CS}GGN6, compared to ^{CS}GGN6, had a considerably increased binding affinity for a neutral lipid, while both peptides showed comparable affinities for negatively charged lipids [49]. Second, the P14A GGN5, unlike GGN5, showed high toxicity to erythrocytes, where the charge interaction between the peptide and lipid head groups would not be critical. Another interesting finding has been reported for the N-terminal fragments of GGN5. The N-terminal 13-residue fragment of GGN5 ($GGN5^{N13}$) still exhibited a high antimicrobial activity but with concomitant hemolytic activity [31]. This result strongly suggested that the C-terminal segment from the proline kink would be critical for suppressing hemolytic activity. However, it cannot be said that the C-terminal segment is unnecessary for antimicrobial activity of GGN5, since the C-terminal substitutions from cysteines to serines significantly impaired the antimicrobial activity. Probably the $GGN5^{N13}$ is expected to possess antimicrobial activity by a different mechanism from that of the intact GGN5.

In summary, in additional to the intrinsic helicity and the Rana box, three other properties of GGN5 and GGN6 are suggested for their structural requirements to properly interact with membranes at least for primary binding: the amphipathicity of the helix, the helical kink by Pro 14, and the N-terminal hydrophobic cluster. The proline kink in membrane-spanning helices has been postulated to aid in the gating mechanism of ion-channel proteins, such as transport proteins and pore-forming antimicrobial peptides [49,62]. However, it has not yet been confirmed whether the peptides finally function via a pore-forming or the carpet-like mechanism. Amphipathic properties are

favorable for both the mechanisms [34–41]. Thus, further complementary investigations would be required to clarify the action mechanism and functional implications of the structural requirements.

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