Origin of Low Mammalian Cell Toxicity in a Class of Highly Active Antimicrobial Amphipathic Helical Peptides*

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We recently described a novel antimicrobial peptide, RTA3, derived from the commensal organism Streptococcus mitis, with strong anti-Gram-negative activity, low salt sensitivity, and minimal mammalian cell toxicity in vitro and in vivo. This peptide conforms to the positively charged, amphipathic helical peptide motif, but has a positively charged amino acid (Arg-5) on the nonpolar face of the helical structure that is induced upon membrane binding. We surmised that disruption of the hydrophobic face with a positively charged residue plays a role in minimizing eukaryotic cell toxicity, and we tested this using a mutant with an R5L substitution. The greatly enhanced toxicity in the mutant peptide correlated with its ability to bind and adopt helical conformations upon interacting with neutral membranes; the wild type peptide RTA3 did not bind to neutral membranes (binding constant reduced by at least 1000-fold). Spectroscopic analysis indicates that disruption of the hydrophobic face of the parent peptide is accommodated in negatively charged membranes without partial peptide unfolding. These observations apply generally to amphipathic helical peptides of this class as we obtained similar results with a peptide and mutant pair (Chen, Y., Mant, C. T., Farmer, S. W., Hancock, R. E., Vasil, M. L., and Hodges, R. S. (2005) J. Biol. Chem. 280, 12316–12329) having similar structural properties. In contrast to previous interpretations, we demonstrate that these peptides simply do not bind well to membranes (like those of eukaryotes) with exclusively neutral lipids in their external bilayer leaflet. We highlight a significant role for tryptophan in promoting binding of amphipathic helical peptides to neutral bilayers, augmenting the arsenal of strategies to reduce mammalian toxicity in antimicrobial peptides.

During the last 15–20 years, the growing problem of resistance to classical antibiotics has focused attention on novel classes of antimicrobial molecules (1–3). One of these is the broad group of antimicrobial peptides that constitute the first line of defense against invading organisms in higher animals (4–8). Two general features of these peptides are that they are amphipathic (adopting conformations that separate polar and nonpolar surface to match the polar-nonpolar interfacial regions of cell membranes) and are positively charged (promoting interaction with the negatively charged membranes of prokaryotic cells). Antimicrobial peptides are effective at low micromolar concentrations against a broad range of microorganisms, including in many cases those resistant to traditional antibiotics (4–8). In general, however, therapeutic applications of these peptides have been hindered by several problems, perhaps the most important being toxicity, cost of production, and bioavailability. Continuing advances in peptide synthesis and purification, combined with economies of scale in large volume syntheses, indicate that the cost of therapeutics based on peptides of relevant size (12–20 amino acid residues) is less of an issue than in the past (9). Bioavailability problems are likely to be overcome with peptides showing genuine promise (for example by using D-amino acid versions of effective peptides (10)). Toxicity, however, remains a limiting factor in peptide antimicrobial use. This is illustrated by the observation that antimicrobial peptides are rarely effective in animal studies at doses below ~10–20 mg kg⁻¹. Because peptides of this class so far cannot be administered orally, this therapeutic dose requires injections of significant volumes of peptide at concentrations of 1 or 2 mg ml⁻¹, corresponding to concentrations in the millimolar range. Thus, although the effective dose as determined by MICs² may be in the low micromolar range, the dosing methods require that peptides have low eukaryotic cell toxicity at rather high concentrations. Partly as a result of these considerations, first generation antimicrobials based on peptides derived from animal or bacterial sources have been limited to topical use (e.g. pexaganin based on magainin from frog skin (11)) or are chemically modified to reduce in vivo toxicity (colistin methanosulfonate in which the active form of the peptide is probably the unmethanosulfonated form resulting from loss of side chain protection in vivo (12)).

A promising approach to the development of safer peptide antimicrobials is the identification of peptides secreted internally in animals (13–15) or from commensal organisms in animals. In these cases the natural evolutionary systems will be expected to have generated effective sequences with low toxicity. Although the direct therapeutic use of endogenous peptides might itself be problematic because of the potential promotion

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2 The abbreviations used are: MIC, minimum inhibitory concentration; CF, carboxyfluorescein; FPE, fluorescein phosphatidylethanolamine; PC, phosphatidylcholine; PG, phosphatidylglycerol; SUV, small unilamellar vesicle.
of resistance against parts of the endogenous immune system (16), the development of peptide antimicrobials incorporating structural themes from endogenous peptides is expected to be useful.

We have recently described such a peptide, RTA3, based on the identification of antimicrobial activity in the commensal organism, Streptococcus mitis.² This peptide (see Fig. 1) was based on a gene sequence discovered while investigating anti-Pseudomonas activity in sputa from cystic fibrosis patients. Although the putative parent peptide, upon synthesis, was highly salt-sensitive, a modification to RTA3 based on systematic alanine scan mutagenesis and sequence changes based on highly charged residue (Arg-5) that falls on the nonpolar face, this residue, in a membrane surface-localized helical peptide (17), can re-position itself in the polar regions of the membrane interface by helix “fraying” that is often encountered in the interaction of interfacially localized helical peptide with membranes (18, 19). To address the role of the Arg-5 residue and the effects of disrupting the nonpolar face of the helical peptide, we have prepared tryptophan-substituted versions of RTA3 (RTA3-F4W) and a variant (RTA3-F4W,R5L) in which the strict amphipathic helical amino acid distribution is recovered, and we tested the membrane binding and biological activities of this peptide (the F4W substitution was introduced to facilitate membrane binding studies). To determine whether the observations on this peptide pair are generally applicable, we have also synthesized a previously described (20) pair of peptides, V₆₈₁ (an antimicrobial amphipathic helical peptide with high eukaryotic cell toxicity), and a mutant, V₆₈₁V₁₃₉ (Fig. 1), that has suppressed eukaryotic cell toxicity upon disruption of the nonpolar helix face, and we tested their interactions with membranes of varying composition. In contrast to previous explanations involving the subtle dispositions of the peptides in membranes of different compositions, we find that disruption of the hydrophobic face of the amphipathic helical peptides in wild type RTA3 and the mutant V₆₈₁V₁₃₉ reduces eukaryotic cell toxicity because of a greatly reduced affinity for neutral membranes, which is particularly marked for RTA3. Surprisingly, each of the peptides with a disrupted hydrophobic surface appears to bind to negatively charged membranes without major disruption of helical conformations to move the positively charged side chain (Arg or Lys) from its apparent loca-

² Hawrani, A., Dempsey, C. E., Howe, R. A., and Walsh, T. R., presented at the 47th ICAAC Meeting, Chicago, IL, September 17–20, 2007, abstracts F1658–F1661.

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**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis, Purification, and Characterization**—The peptides listed in Table 1 were synthesized by Dr. G. Bloomberg of the Bristol Centre for Molecular Recognition using standard Fmoc (N-(9-fluorenyl)methoxycarbonyl) solid phase synthesis. The peptides were purified by high pressure liquid chromatography and were confirmed to be at least 97% pure by analytical high pressure liquid chromatography and to have the predicted m/e ratio by mass spectrometry. Phospholipids were from Lipid Products (Nutfield, UK); carboxyfluorescein (CF) was from Sigma, and fluorescein-phosphatidylethanolamine (FPE) was from Avanti Polar Lipids.

**Biological Activities**—MICs of the peptides were determined by broth microdilution according to the Clinical and Laboratory Standards Institute (21). 100 µl of 0.5–1 × 10⁶ colony-forming units per ml of Pseudomonas aeruginosa in Mueller Hilton media (plus cations) broth (BD Biosciences) were incubated in 96-well microtiter plates with serial 2-fold dilutions of the peptides. MICs were defined as the lowest peptide concentration with no visible growth of bacteria from the MIC microtiter plates after 24 h at 37 °C. Hemolytic activity was determined by incubating 10% (v/v) suspension of horse erythrocytes with peptides. Erythrocytes were rinsed in 10 mM phosphate-buffered saline, pH 7.2, by repeated centrifugation and resuspension (3 min at 3000 × g). Erythrocytes were incubated at room temperature for 1 h in either deionized water (fully hemolyzed control), phosphate-buffered saline, or with peptide in phosphate-buffered saline. After centrifugation at 10,000 × g for 5 min, the supernatant was separated from the pellet and the absorbance measured at 570 nm. Absorbance of the suspension treated with deionized water defined complete hemolysis.

**Preparation of Lipid Vesicles**—All experiments were performed at room temperature. Small unilamellar vesicles (100 nm diameter) were used for all spectroscopic measurements except for CD spectroscopy for which smaller (50 nm) vesicles were used to minimize light scattering effects. Lipids were dried from chloroform:methanol solution and pumped under high vacuum overnight to remove traces of solvent. Dried lipids were hydrated at a concentration of 10 mg ml⁻¹ in 10 mM Tris-HCl, pH 7.4, containing either 107 mM NaCl (buffer A) or, for the CF dye-release experiments, 50 mM CF. Vesicles doped with FPE were prepared similarly except that 0.5 mol % of FPE in methanol was added to the lipids in organic solvent before drying. Hydrated lipids were extruded 10 times through two 100 or 50 nm pore membranes, using a Lipex Biomolecular extruder (Vancouver, Canada). Vesicles for CD and peptide binding, monitored using either tryptophan fluorescence or FPE fluorescence, were used directly. Vesicles for CF dye-release measurements were used after gel filtration on a Sephadex G-15 column with buffer A as the mobile phase, to remove non-trapped CF. Thus in all experiments, interaction of the peptide with vesicles was determined in the same buffer (buffer A).
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Fluorescence Spectroscopy—Fluorescence measurements were made using a SPEX Fluoromax fluorimeter. Peptide solutions were made in plastic tubes or cuvettes to minimize loss of peptide at low concentrations because of binding to glass surfaces. For the measurement of vesicle-induced changes in the emission spectra of tryptophan in Trp-containing peptides, a 2 μM peptide solution was incubated in buffer A, and aliquots of vesicles suspension were added to give total lipid concentrations in the range 0 to 300 μM total lipid. Tryptophan fluorescence was excited at 280 nm, and the emission spectrum was measured between 300 and 450 nm in 1-nm increments with 1-s signal averaging. Binding data were fitted to a simple hyperbolic function to obtain estimates of the maximum fluorescence emission blue shift (Δλ_{max}) and the concentration of lipid at which the lipid-induced blue shift was half-maximal.

Peptide binding to FPE-labeled vesicles was measured by adding aliquots of peptide to a suspension of vesicles at 65 μM total lipid concentration in buffer A. FPE emission was measured at 520 nm (excitation at 490 nm). The experiments were made by adding successive aliquots of peptide to a single vesicle sample. Control experiments showed that the same FPE fluorescence enhancement was obtained by adding a single large aliquot of peptide or the same amount of peptide in successive small aliquots; the latter method facilitates analysis of peptide binding at low peptide concentrations allowing an assessment of the extent to which binding is cooperative (22).

Peptide-induced dye release from vesicles loaded with CF was measured from the loss of CF self-quenching as the dye dilutes into the extravascular medium. Experiments were done with the same lipid concentration (65 μM) as the FPE binding measurements and in buffer A so that data from the different experiments can be interpreted in a consistent manner. CF emission was measured at 520 nm (excitation at 490 nm). The fluorescence resulting from 100% release of encapsulated CF was determined by adding 10 μl of 20% Triton X-100. To ensure rapid mixing of peptide and vesicles, and to avoid high local concentrations of peptide, 1 ml of a peptide solution at double the post-mix concentration was rapidly ejected from an Eppendorf pipette into 1 ml of a vesicle suspension at 130 μM concentration (65 μM post mix) to initiate binding and dye release. The fluorescence emission intensity was measured 3 min after mixing CF-loaded vesicles with peptide.

Circular Dichroism Spectroscopy—CD spectra were obtained at 20 °C using a Jobin-Yvon CD spectropolarimeter. All samples were made in buffer A. Spectra of peptides in solution were measured in 1- or 2-mm quartz cuvettes. Spectra in the presence of vesicles were measured in 0.1-mm path length cuvettes to minimize light scattering contributions. All spectra are averages of 5 (vesicle-free solutions) or 9–11 scans (peptides plus vesicles) with appropriate peptide-free blank spectra subtracted and were zeroed at 260 nm before plotting without smoothing. Peptide helix content was calculated from the ellipticity at 222 nm (θ_{222}) (23) using parameters determined by Luo and Baldwin (24).

RESULTS

Peptide Nomenclature and Biological Activities of Peptides

The experiments described below were designed to compare the membrane binding properties of two sets of peptides, each set comprising a peptide(s) having the nonpolar helix face disrupted by a positively charged Arg or Lys residue (RTA3-dis; WRTA3-dis and V681-dis), and the other of the set having a non-disrupted helix face (WRTA3-non-dis and V681-non-dis). We have employed the "dis"/"non-dis" designation here to simplify the terminology of these peptides, particularly because the "wild type" RTA3 peptide (RTA3-dis) has a disrupted (dis) nonpolar helix face, whereas it is the "mutant" V681 peptide (V681-V13K or V681-V681) that has the disrupted (dis) nonpolar helix face. We synthesized F4W variants of the RTA peptides (WRTA3-dis and WRTA3-non-dis), in which the Phe-4 residue was replaced with a tryptophan, for two reasons. First, to obtain accurate peptide concentrations that are important for quantitative interpretation of CD data. Second, the presence of a tryptophan residue allows quantitative analysis of peptide binding to lipid vesicles through the effects of binding on tryptophan fluorescence. V681 already contains a tryptophan (Trp-2) in its "native" sequence (Fig. 1).

The minimum inhibitory concentrations against *P. aeruginosa* and the hemolytic activities of the peptides are given in...
Table 1 and Fig. 2. We present hemolysis data up to very high concentrations that are relevant for in vivo dosing considerations as described in the Introduction.

Peptides with disrupted nonpolar helical faces have greatly reduced hemolytic activity while retaining anti-Pseudomonas activity similar to that of the equivalent peptide having a non-disrupted nonpolar helix face. The latter peptides have very high hemolytic activity. These observations are generally consistent with the results on V681 and V681-V13K previously described by Chen et al. (20). Two important observations that we explore below are the extremely low hemolytic activity of wild type RTA3 at very high concentrations, and the significantly enhanced hemolytic activity of the analog of RTA3 in which the Phe-4 residue is replaced with a tryptophan (Table 1; Fig. 2).

Conformational Transitions upon Membrane Binding

Each of the peptides in Fig. 1 is unstructured in buffer A as indicated by a “random coil” CD spectrum (not shown). Fig. 3A shows that both WRTA3-dis and WRTA3-non-dis adopt helical structure upon binding to membrane vesicles composed of 50% PC and 50% PG in buffer A. The helical content was around 78% in each case, which is very similar to the maximum helical content induced by these peptides in 40% trifluoroethanol (in buffer A; data not shown), which generally induces a maximum helix content in peptides with moderate to high helix-forming potential (24). The high concentrations of peptide (150 μM) and total lipid concentrations were 10 mM. For both panels, filled symbols denote peptide spectra in the presence of vesicles composed of PC and PG (50:50, mol/mol). Squares are peptides with nondisrupted helical faces (WRTA3-non-dis in A, and V681-non-dis in B); circles are peptides with disrupted helical faces (WRTA3-dis in A, and V681-dis in B), and RTA3-dis is denoted by triangles.
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type RTA3) when incubated with high concentrations of vesicles composed of neutral lipid (Fig. 3A). This observation indicates that the apparently conservative replacement of the Phe-4 residue of RTA3-dis with a Trp results in measurable differences in the interaction of RTA3-dis and WRTA3-dis with neutral membranes.

Similar observations were made with the wild type V681-non-dis and V681-dis mutant (V681-V13K) that has the disrupted nonpolar helix face (Fig. 3B). Each of the peptides has a high helical content induced upon incubation with negatively charged membranes, but only the V681-non-dis wild type peptide, having an intact nonpolar helix face (Fig. 1), has a high helix content induced upon interaction with membranes composed of neutral lipids. The V681-dis peptide has slightly reduced helical content when bound to negatively charged membranes (68%) compared with the wild type V681-non-dis (81%) indicating that helical conformations are somewhat disrupted upon binding at the membrane interface. However, because 81% helix corresponds to around 21 amino acid residues in V681 and 68% corresponds to 17–18 amino acid residues, the V13K mutation does not result in large scale unfolding of helical conformations to release the Lys-13 residue from the nonpolar helix face.

Membrane Binding

*Trp Fluorescence*—The sample restrictions in CD spectroscopy require that conformational analyses that relate to membrane binding can only be done under conditions of very high lipid and peptide concentrations. In addition, the possibility that the absence or limited amount of helical conformations in the presence of lipid vesicles results from binding without structure formation cannot be formally ruled out from CD data alone. We therefore performed two separate series of experiments to assess peptide binding directly, and under conditions that more closely match the peptide concentrations used in antimicrobial assays. These experiments allow an assessment of relative membrane binding affinities of the peptides, and additionally can establish whether binding is cooperative or exhibits “hyperbolic” behavior. These are each of interest with respect to the mechanisms of the membrane properties of the peptides. Because RTA3 does not contain a Trp residue, we used F4W “mutants” to assess binding to phospholipid vesicles based on binding-induced perturbation of Trp fluorescence.

The sequestering of the Trp indole group in an environment of reduced polarity upon membrane binding results in a blue shift in the fluorescence excitation maximum, as illustrated by the emission spectrum of the Trp-2 residue of V681-dis when titrated with increasing concentrations of lipid in the form of vesicles composed of 50% PC:PG (Fig. 4A). Titrating the same peptide with neutral lipid vesicles (100% PC) results in very small lipid-dependent blue shifts (Fig. 4B). Fig. 5 illustrates the lipid concentration-dependent fluorescence blue shifts of each of the tryptophan-containing peptides. Very similar behavior was observed for the RTA3 (Fig. 5A) and V681 (Fig. 5B) series of peptides. In each case disruption of the nonpolar helix face (with an Arg (WRTA3-dis) or Lys (V681-dis)) had only a minor effect on peptide binding to negatively charged vesicles, whereas binding to neutral vesicles was greatly suppressed. An intact nonpolar helix face (WRTA3-non-dis, V681-non-dis) resulted in strong binding to neutral lipid vesicles, although the reduced maximum blue shift upon saturation indicates that the tryptophan indole in each peptide may adopt a shallower membrane insertion compared with the binding to negatively charged vesicles. Likewise the reduced Trp-4 blue shift of WRTA3-dis, compared with WRTA3-non-dis, on binding to negatively charged membranes may also indicate a location of Trp-4 closer to the membrane surface for the former peptide compared with the latter.

*Fluorescein-Phosphatidylethanolamine (FPE) Fluorescence*—The very small structure-formation in WRTA3-dis (Fig. 3A) and V681-dis (Fig. 3B) upon incubation with vesicles composed of neutral lipids, and the very small perturbation of Trp fluorescence induced by neutral lipid vesicles (Figs. 5, A and B) support the conclusion that these peptides bind very poorly to membranes lacking a negative surface charge. However these experiments do not formally rule out the possibility that the peptides might bind with neither structure formation nor burial of the respective Trp res-
disrupts the highly positively charged antimicrobial peptides, and it is a useful way of assessing cooperativity in peptide binding under conditions that do not involve extremely high peptide:lipid ratios at the initial parts (low lipid concentration) of the binding curves determined by titrating a fixed concentration of peptide with increasing lipid concentrations.

**FIGURE 6.** FPE fluorescence enhancement resulting from RTA3 peptides binding to PC:PG (50:50, mol/mol) (A) or 100% PC vesicles (B). The total vesicle lipid concentration was 65 μM in all cases, and the buffer was 10 mM Tris-HCl, 107 mM NaCl, pH 7.4 (20 °C). Circles (RTA3-dis) and triangles (RTA3-dis) represent peptides with disrupted nonpolar helix faces, and squares are WRTA3-non-dis (nondisrupted nonpolar helix face).

**FIGURE 7.** FPE fluorescence enhancement resulting from V681 peptides binding to PC:PG (50:50, mol/mol) (A) or 100% PC vesicles (B). The total vesicle lipid concentration was 65 μM in all cases, and the buffer was 10 mM Tris-HCl, 107 mM NaCl, pH 7.4 (20 °C). Circles represent V681-dis (disrupted nonpolar helix face), and squares are V681-non-dis (intact nonpolar helix face).

Consistent with the tryptophan fluorescence binding data, neutral vesicles strongly distinguish between peptides having intact and disrupted nonpolar helix faces (Fig. 6B). The binding of WRTA3-non-dis to PC vesicles is nearly as strong as binding to PC:PG vesicles, whereas WRTA3-dis binds very weakly. Reverting WRTA3-dis back to the wild type peptide (RTA3-dis) virtually abolishes binding to neutral vesicles. In fact, the wild type peptide with a perturbed nonpolar helix face and lacking a tryptophan residue has a binding constant for neutral membranes reduced more than 1000-fold compared with its interaction with negatively charged membranes. This observation explains the difference in structuring between RTA3-dis and WRTA3-dis on incubating with very high concentrations of neutral vesicles (Fig. 3A).

The FPE vesicle binding data for the V681 peptides (Fig. 7) are generally consistent with those described for the RTA3 peptides (Fig. 6) and for V681 peptides binding measured using tryptophan fluorescence (Fig. 5B). We were only able to determine binding of wild type V681 (V681-non-dis) to PC:PG vesicles at moderate peptide concentrations because interaction of the peptide with these vesicles at higher concentrations induced light scattering because of peptide-induced vesicle aggregation or fusion. However, the data demonstrate rather similar binding of V681-non-dis and V681-dis to negatively charged vesicles (Fig. 6A). Likewise the FPE binding data confirm that the minimal structuring of V681-dis upon incubation with neutral lipid vesicles (Fig. 3B) and the limited effect of neutral vesicles on the fluorescence emission of the Trp2 residue (Fig. 5B) is because of low binding affinity of this peptide to neutral membranes.
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For all of the peptides studied, no evidence of cooperative binding (either positive or negative cooperativity) was apparent, because in each case linear variations in fluorescence enhancement with increasing peptide concentrations were observed at low peptide concentrations.

Membrane Perturbation, Vesicle Dye Release

How do the membrane binding properties of the peptides relate to their effects on the structural integrity of phospholipid membranes? We tested the ability of the peptides to release CF trapped at high concentrations within 100 nm SUV composed of either 50% PC:PG or 100% PC under conditions (65 μM total lipid, high salt buffer) equivalent to those used for the FPE binding experiments of Fig. 7. All of the data are compiled in Fig. 8.

Each of the five peptides releases internal CF from negatively charged vesicles at low peptide concentrations, either through pore formation or generalized disruption of the lipid membrane bilayer organization (Fig. 8A); notice that the designation of the peptides is different in Fig. 8, with open and closed symbols referring to RTA3 and V681 peptides, respectively. The membrane lytic activity generally relates to the positive charge density of the peptides with the highly charged (5 or 6 positive charges) V681-V13K having the greatest activity. Disruption of nonpolar helical faces (WRTA3-non-dis and V681-non-dis) had very high activity against neutral lipid vesicles with half-maximal activities in the range 0.3–0.5 μM (Fig. 8B). Consistent with the membrane binding data of Figs. 5–7, peptides with nonpolar helix faces disrupted with a positively charged residue had very low activity against neutral vesicles. Wild type RTA3 (RTA3-dis) was particularly ineffective against neutral vesicles with barely detectable dye release (1–2%) at a concentration of 50 μM. This corresponds to a ratio of effectiveness in dye release from negatively charged vesicles over neutral vesicles of around 10,000-fold, a factor that results from a combination of the greatly reduced binding of RTA3 to neutral membranes (near 1000-fold) and the apparent cooperativity in peptide-induced membrane perturbation.

Summary of Peptide Membrane Interactions

The membrane-induced helix formation, membrane binding, and membrane perturbation data for the five peptides studied is summarized in Table 2. In all experiments, peptides having nonpolar helix surface disrupted with a positively charged Lys (V681-V13K) or Arg residue (RTA3, RTA3-F4W) showed limited binding to neutral lipid vesicles coupled with very low activity in disrupting vesicles composed of neutral lipids. On the other hand, these peptides were very active against negatively charged vesicles.

TABLE 2

| Membrane-induced structure | Membrane binding | Bilayer disruption |
|-----------------------------|------------------|-------------------|
| CD (% helix)                | Trp fluorescence | FPE fluorescence | CF dye release |
|                             | PC | PC:PG | PC | PC:PG | PC | PC:PG | PC | PC:PG | (μM) |
| RTA3                        | <5 | >75  | >3000 | 3.2  | 735 | 0.068 | 2.2 |
| RTA3-F4W                    | 10 | 78   | 196  | 3.2  | 30  | 0.073 | 1.8 |
| RTA3-F4W,R5L                | 78 | 82   | 2.2  | 4.3  | 0.11| 0.065 | 2.0 |
| V681                        | 82 | 81   | 2.0  | 1.7  | 0.37| 0.51  | 2.3 |
| V681-V13K                   | 12 | 68   | 125  | 1.4  | 63  | 0.27  | 2.2 |

a The % peptide helix was measured under the following experimental conditions: 150 μM peptide, 10 mM total lipid (50 nm SUV) in 10 mM Tris, 107 mM NaCl, pH 7.4 (buffer A).

b The lipid concentration (as 100 nm SUV) that induced 50% peptide binding; the peptide concentration was 2 μM in buffer A.

c The peptide concentration that induced a half-maximal FPE fluorescence shift in FPE-doped 100 nm SUV (65 μM total lipid) in buffer A.

d The peptide concentration required for half-maximal CF dye release from 100 nm SUV (65 μM total lipid) in buffer A.

* The number in parentheses is the value of n in the sigmoidal fit of dye release data (see legend to Fig. 8).
peptides retain strong binding to negatively charged vesicles and perturb these membranes (either via nonspecific membrane perturbation or “pore” formation) at low concentrations. This general observation is entirely consistent with the relative activities of the peptides on eukaryotic cells (erythrocyte hemolysis) and bacterial cells (MICS), respectively (Fig. 2 and Table 1).

We have tabulated the data in Table 2 in terms of the lipid or peptide concentration required to elicit half-maximal binding or half-maximal dye release. This is appropriate for the dye-release data because the dose-response curves (Fig. 8) in PC:PG are sigmoidal with near second-order dependence on peptide concentration for each of the peptides studied (Table 2). The binding data fitted simple hyperbolic binding isotherms in all cases, indicating that any cooperativity in peptide-induced dye release in PC:PG vesicles must arise from the properties of the membrane-bound peptides, rather than in the binding process itself.

Although the membrane binding measured by Trp fluorescence (titrating peptide with increasing vesicular lipid concentration) and using FPE-doped membranes (titrating vesicular lipid with increasing peptide concentration) probes a single binding equilibrium for any particular peptide:lipid composition pair (e.g. RTA3 binding to pure PC membranes), the FPE and Trp fluorescence data cannot be combined and fitted to a single equilibrium constant, because the peptide-membrane interaction corresponds to a partitioning rather than a discrete site-binding phenomenon (26). At the very least, one would need to know the number of lipids that constitute a “peptide-binding site” to make this analysis; however, the membrane is essentially a continuous “homogeneous” surface that cannot formally be dissected into “binding sites.” On the other hand, the Trp fluorescence data can be used to determine a mole fraction partition coefficient ($K_x$), according to Equation 1, that quantitates the partitioning of peptide between the aqueous and membrane phases (26), and these values are useful for comparison with previous studies of peptide-membrane partitioning.

$$\Delta \lambda = \frac{\Delta \lambda_{\text{max}} K_x [L]}{[W] + K_x [L]}$$  (Eq. 1)

In Equation 1, $\Delta \lambda$ and $\Delta \lambda_{\text{max}}$ are the lipid-induced shift in the peptide Trp fluorescence emission wavelength, and its maximum value, respectively; [L] is the lipid concentration, and [W] is the concentration of water (55.3 mM). For the strongly partitioning peptides, $K_x$ is in the range of $2 \times 10^4$ to $1.5 \times 10^5$, whereas for the weakly binding systems these are 2 orders of magnitude or more smaller (estimated to be less than $5 \times 10^4$ and less than $2 \times 10^4$ for RTA3-F4W/PC; V$_{681}$-V13K/PC, respectively). These values can be compared, for example, to the binding of magainin to PC:PG (50:50) ($K_x = 5.4 \times 10^6$, in a similar high salt buffer to that used here (22)) and of melittin binding to neutral vesicles ($K_x \sim 10^5$ (27)). $K_x$ in this analysis, cannot be measured for peptides lacking a Trp residue but must be well below $10^4$ for RTA3 binding to neutral membranes, because this peptide binds at least 10 times more weakly than RTA3-F4W and V$_{681}$-V13K as measured by FPE fluorescence (Table 2).

**Lipid Composition Dependence of RTA3 Peptide Activities**

The binding and membrane-disrupting effects of V681 and RTA3 peptides on PC and PC:PC (50:50) membrane vesicles broadly correlate with their effects on mammalian (erythrocytes) and bacterial (P. aeruginosa) cells, respectively. To determine whether these observations apply to other lipid compositions, we measured RTA3-induced membrane binding and trapped dye release using 100 nm SUV having lipid compositions more similar to bacterial and mammalian membranes. These lipid compositions were PE:PG:cardiolipin (80:10:15) as an analog of bacterial cell membranes (28), and PC:PE:phosphomyelin:cholesterol (35:30:25:10), as an analog of erythrocyte membranes (28, 29).

These data are compiled in Table 3, and binding and dye-release data with the erythrocyte-mimetic membrane vesicles are shown in Fig. 9. The RTA3 peptides show strong binding to membranes having lipid compositions similar to those of
bacterial membranes, although the binding is reduced by 3–5-fold compared with binding to PC:PG (50:50) membranes (Table 3), probably because of the reduced negative surface charge in membranes containing 20–25%, rather than 50%, negatively charged lipids. Likewise, the binding and membrane perturbation of RTA3 peptides containing disrupted nonpolar helix surfaces (RTA3 and RTA3-F4W) is negligible when tested against PC:PE:sphingomyelin:cholesterol (35:30:25:10). Indeed, these RTA3 peptides bind even less strongly and are less effective in perturbing the bilayer membrane integrity, when tested against vesicles having the latter lipid composition, compared with the effects on pure PC membranes. This reduced binding affinity may be due to the effect of cholesterol in reducing the fluidity of the bilayer membrane and a suppression of peptide partitioning into the membrane interfacial region (30). The replacement of Arg-5 of RTA3 with Leu-5 in RTA3-R5L restores strong binding and dye release (Fig. 9) consistent with the observations described in Figs. 2, 3, and 5–8 and Table 2.

**DISCUSSION**

This study illustrates that vesicle bilayer membranes composed either of neutral lipids or mixed PG:PC membranes can be surprisingly good analogs for eukaryotic and bacterial cell membranes, respectively, consistent with a body of work that highlights membrane surface charge as a dominant feature in the selectivity of positively charged amphipathic peptides for bacterial membranes (5, 30–33). These observations also apply to more complex lipid mixtures designed to match the lipid compositions of bacterial and mammalian cell cytoplasmic membranes. The general correspondence between the effects of the peptides on vesicles (Figs. 8 and 9; Tables 2 and 3) and on bacteria or erythrocytes (Fig. 2 and Table 1) provides strong evidence that interaction with the membranes of target cells plays an important role in their mechanisms of antimicrobial action. A detailed analysis of vesicle binding, membrane-induced structure formation, vesicle membrane perturbation, and activity measurements against target bacteria and erythrocytes yields a consistent interpretation of the effects of disruption of the amphipathic structure of positively charged antimicrobial helical peptides by inserting a positively charged amino acid onto the nonpolar helix face. As described previously (20), this modification can result in greatly reduced eukaryotic cell toxicity while retaining high antimicrobial activity, a conclusion that is reinforced by similar observations with RTA3 and the designed sequence variants (Fig. 2; Table 1). It was previously suggested that the reduction of activity against eukaryotic membranes upon disruption of the nonpolar helix face (with a Lys in V681-V13K) resulted from the inability of the peptide to insert deeply into the cell membrane to form peptide “poles” (20). Our results demonstrate that the loss of membrane disruption of neutral membranes, including those of erythrocytes, has a simpler explanation; the peptides simply do not bind to membranes without negative surface charge.

This conclusion is generally consistent with our understanding of the interaction of amphipathic helical peptide with the interfacial region of bilayer membranes. For neutral membranes, binding is dominated by the interaction of the amphipathic peptide structure with the complementary membrane interfacial region. Amphipathic helix formation is crucial for this process because it allows the polar peptide backbone to substitute intramolecular (helical) hydrogen bonding for the loss of solvation energy as the peptide buries within the interfacial region of the bilayer (34). If the amphipathic structure is perturbed, this essential contribution to binding is lost. If the peptide helix partially unwinds to remove the positively charged amino acid from the nonpolar helix face, then the amphipathic nature of the peptide that dominates the favorable binding energy is again attenuated. Thus nonampipathic peptides cannot easily bind in the interfacial region of neutral bilayer membranes. The results shown here demonstrate that a single positively charged amino acid (Lys or Arg) on the nonpolar face of an interfacially bound amphipathic helical peptide is sufficient for very large attenuation of binding to neutral phospholipid bilayer membranes, a result that is consistent with the very high energetic barriers to the burial of a positively charged residue in the nonpolar regions of a membrane (35).

On the other hand, binding to negatively charged membranes has contributions both from the complementary nature of helical amphipathic peptides and the interfacial region of the bilayer, and complementary electrostatics. As indicated by the CD data of Fig. 3, the negatively charged membrane surface may also provide complementary negative charges for the positively charged amino acids disrupting the nonpolar helix face. This seems to be required to explain the observation of the retention of virtually unperturbed helical content of RTA3 and RTA3-F4W, and the relatively small perturbation of helical structure in V681-V13K, on binding to negatively charged membranes. Observations of RTA3-induced vesicle fusion, together with molecular dynamics simulations, suggest that the accommodation of the Arg residue on the nonpolar helix surface of RTA3 may be achieved by tilting of the peptide helix at the membrane interface that allows the Arg-5 side chain to reach the well solvated regions of the interface. However, the accommodation of the Lys-13 residue on the nonpolar helix face of V681-V13K upon binding to negatively charged membranes is unlikely to be achieved by helix tilting because the positively charged residue lies near the center of the relatively long peptide helix, and an unrealistically high degree of helix tilt would be required for the Lys residue to “reach” the highly hydrated regions of the membrane interface. In the case of V681-V13K, lipid head group phosphates might provide “neutralizing” charges for the positive Lys-13 charge.

An unexpected finding is the observation that substitution of Phe-4 of RTA3 with Trp significantly enhances binding to neutral membranes. In hindsight, this is understandable in terms of the very high interfacial propensity of Trp compared with all the other amino acids, even though Phe is a more “hydrophobic” amino acid side chain (36). This observation indicates that the removal of Trp residues from amphipathic helical antimicrobial peptides might be an additional general strategy for reducing eukaryotic cell toxicity. The absence of a tryptophan, combined with the presence of an arginine residue on the non-

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polar helix face, results in a peptide with extraordinarily low binding to neutral lipid membranes, and this seems to underlie the extremely low eukaryotic cell toxicity in RTA3, despite the retention of very high affinity for binding to negatively charged membranes (Table 2) and strong antimicrobial activity (Table 1). These specific sequence characteristics are a product of evolutionary design within a commensal organism (S. mitis) and highlight the potential of commensals as a source of novel antimicrobials having low eukaryotic cell toxicity.

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