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Amphipathic α-helical peptide, HP (2–20), and its analogues derived from *Helicobacter pylori*: Pore formation mechanism in various lipid compositions

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Abstract

In a previous study, we determined that HP(2–20) (residues 2–20 of parental HP derived from the N-terminus of *Helicobacter pylori* Ribosomal Protein L1) and its analogue, HPA3, exhibit broad-spectrum antimicrobial activity. The primary objective of the present study was to gain insight into the relevant mechanisms of action using analogues of HP(2–20) together with model liposomes of various lipid compositions and electron microscopy. We determined that these analogues, HPA3 and HPA3NT3, exert potent antibacterial effects in low-salt buffer and antifungal activity against chitin-containing fungi, while having little or no hemolytic activity or cytotoxicity against mammalian cell lines. Our examination of the interaction of HP(2–20) and its analogues with liposomes showed that the peptides disturb both neutral and negatively-charged membranes, as demonstrated by the release of encapsulated fluorescent markers. The release of fluorescent markers induced by HP(2–20) and its analogues was inversely related to marker size. The pore created by HP(2–20) shows that the radius is approximately 1.8 nm, whereas HPA3, HPA3NT3, and melittin have apparent radii between 3.3 and 4.8 nm. Finally, as shown by electron microscopy, the liposomes and various microbial cells treated with HPA3 and HPA3NT3 showed oligomerization and blebbing similar to that seen with melittin, while HP(2–20) exhibited flabbiness. These results suggest that HP(2–20) may exert its antibiotic effects through a small pore (about 1.8 nm), whereas HPA3 and HPA3NT3 formed pores of a size consistent with those formed by melittin.

Keywords: HP(2–20); Mechanism of action; Fluorescent markers; Oligomerization; Pore-forming

1. Introduction

Membrane-active peptides, including a host of antimicrobials and toxins, have been shown to induce the formation of transmembrane pores. Although the molecular mechanisms by which pore formations occur remain to be thoroughly elucidated, the three mechanisms thus far proposed include the "barrel-stave", "carpet", and "toroidal" models. In the barrel-stave model, peptide helices form a bundle with a central lumen within the membrane, appearing similar to a barrel in which the helical peptides function as the staves [1–5]. This variety of trans-

Abbreviations: BS^3, Bis(sulfosuccinimidyl)suberate; CH, cholesterol; CL, cardiolipin; DCC, dicyclohexylcarbodiimide; Dis-C_3-5, 3,3′, diethylthio-dicarbocyanine iodide; FITC-D, fluorescein isothiocyanate dextrans; HOBT, 1-hydroxy benzotriazole; LPS, lipopolysaccharide; NMP, N-methyl-2-pyrrolidone; PC, egg yolk L-α-phosphatidylcholine; PE, L-α-phosphatidylethanolamine; PG, L-α-phosphatidyl-DL-glycerol; PS, Phosphatidylserine; SM, Sphingomyelin

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membrane pore is induced by treatment with compounds such as alamethicin. It was reported that alamethicin produced pores of ~1.8 nm and ~4.0 nm (inner and outer diameter, respectively) at the threshold concentration of the peptide/lipid ratio [6,7]. These dimensions imply that the walls of the channel are ~1.1 nm in thickness, while is similar to the diameter of the alamethicin helix. Thus, its pores are consistent with an arrangement of eight alamethicin monomers in a barrel-stave configuration [1,8,9].

The carpet model elucidates the activity of antimicrobial peptides such as ovispirin [10]. In this case, peptides accumulate on the bilayer surface, oriented in parallel – i.e., in-plane – with the membrane surface [11,12]. The peptides are electrostatically attracted to the anionic phospholipid head groups at numerous sites, and thereby cover the surface of the membrane in a carpet-like manner [13–17].

In the toroidal-pore model, which has been advanced to explain the activity of the magainins [18], protegrins [19], and melittin [1], the polar faces of the peptides associate with the polar head groups of the lipids and remain in this orientation even when they are perpendicularly inserted into the lipid bilayer, a feature which distinguishes it from the barrel-stave model. The magainin-induced toroidal pores are larger and vary in size to a greater extent than has been observed with the alamethicin-induced pores [1]. They evidence an inner diameter of 3.0–5.0 nm and an outer diameter of ~ 7.0–8.4 nm, and each of the pores is thought to harbor 4–7 magainin monomers and ~90 lipid molecules [1,20]. Melittin, magainin, and alamethicin are the prototype peptides employed in studies of the mechanisms underlying pore formation in both the toroidal and barrel-stave models [21].

Stomach mucosa that is infected with *Helicobacter pylori*, the bacterial pathogen associated with gastritis and peptic ulcers, typically shows massive infiltration of inflammatory cells and tissue destruction [22]. Persistence of *H. pylori* in the mucosa has been suggested to be facilitated by *H. pylori*-produced cecropin-like peptides with antibacterial properties. Although *H. pylori* itself is resistant to this peptide, the release of these peptides gives a competitive advantage over other microorganisms [23].

The linear antimicrobial peptide, HP(2–20), is a cationic α-helical peptide that has been isolated from the N-terminal region of the *Helicobacter pylori* ribosomal protein, L1 [24]. This peptide possesses several important functional characteristics; it is bactericidal, it is a neutrophil chemoattractant, and it activates phagocyte NADPH oxidase to produce reactive oxygen species [24].

The primary objective of the present study was to characterize the effects of HP(2–20) and its analogues on a variety of lipid compositions and cell wall components, and to make observations via electron microscopy. In addition, we assessed pore sizes in these zwitterionic vesicles via dextran leakage and transmission electron microscopy.

2. Materials and methods

2.1. Materials

Proteinase K, trypsin, chitin, cellulose, chitosan, curdlan, peptidoglycan (from *Staphylococcus aureus*), LPS (lipopolysaccharide)[1] (from *Escherichia coli* 0111:B4), BCA protein reagent, BS[3] (Bis(sulfosuccinimidyl)suberate), PE (L-α-phosphatidylethanolamine) (Type V, from *E. coli*), SM (sphingomyelin, from bovine brain), CH (cholesterol, from porcine liver), and FITC-D (fluorescein isothiocyanate dextrans), with average molecular masses of 4, 10, 20, 40, 70, and 500 kDa were all purchased from the Sigma Chemical Co. (St. Louis, MO). PG (L-α-phosphatidyl-DL-glycerol), PC (egg yolk L-α-phosphatidylcholine), PS (phosphatidylserine, from brain) and CL (cardiolipin, from *E. coli*) were obtained from Avanti Polar Lipids (Alabaster, AL). $DiSC_3$-5 (3,3′-diethylthio-dicarbocyanine iodide) and calcein were acquired from Molecular Probes (Eugene, OR). All other reagents were of analytical grade. Buffers were prepared in double glass-distilled water.

2.2. Peptide synthesis and purification

All peptides were synthesized via solid-phase methods with Fmoc (N-(9-fluorenyl)methoxycarbonyl)-protected amino acids on an Applied Biosystems Model 433A peptide synthesizer. 4-Methyl benzhydrylamine resin (Novabiochem) (0.55 mmol/g) was employed to create the amidated C-terminus. For each coupling step, the Fmoc-protected amino acid and coupling reagents were added in a 10-fold molar excess with regard to resin concentration. Coupling (60–90 min) was conducted with DCC (dicyclohexylcarbodiimide) and HOBT (1-hydroxy benzotriazole) in NMP (N-methyl-2-pyrrolidone). Cleavage from the resin and the deprotection of the synthesized peptide were conducted using a solution of 90% trifluoroacetic acid, 3% water, 1% triisopropylsilane and 2% each of 1,2-ethanedithiol, thioanisole, and phenol. After repeated ether precipitation, the crude peptide was purified via reversed-phase preparative HPLC on a Waters 15-μm Deltapak C18 column (19×300 mm) using an appropriate 0–60% acetonitrile gradient in 0.1% trifluoroacetic acid. The purity of the purified peptide was then determined via analytical reversed-phase HPLC using a Vydac C18 column (4.6×250 mm, 300 Å, 5 nm). The molecular masses of the peptides were verified with a matrix-assisted laser desorption ionization mass spectrometer (MALDI II, Kratos Analytical Ins.).

2.3. Antimicrobial assay

Candida albicans was cultured at 28 °C in YPD broth. *E. coli* and *S. aureus* were cultured at 37 °C in trypticase soy broth. The antimicrobial activity of each peptide was determined via microdilution assays. In brief, the microorganisms were collected in mid-log phase and suspended in buffer I (low ionic strength buffer; 10 mM sodium phosphate, pH 7.2) or buffer II [high ionic strength buffer; PBS (1.5 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 135 mM NaCl, pH 7.2)]. Two-fold serial dilutions of each of the peptides, in a range from 0.39 to 200 μM, in buffers I and II, were arranged in sterile 96-well plates, after which aliquots of the cell suspension (1×10^6 CFU/ml) was added to each well. Plates containing fungal cells were incubated for 2 h at 28 °C, while the plates containing the bacterial cells were incubated at 37 °C for the same amount of time. At the end of the incubation, 50 μl of 20-fold diluted samples were plated on appropriate agar plates, and were then incubated for 24 h, after which the colonies were counted. The lowest concentration of peptide that completely inhibited growth was defined as the MIC. The MIC values were calculated as an average of several independent experiments conducted in triplicate.

2.4. hRBC (human red blood cell) hemolysis

Hemolytic activities were assessed for all peptides, using hRBCs from healthy donors, and collected on heparin. The fresh hRBCs were rinsed three times in PBS via 10 min of centrifugation at 800×*g*, and resuspended in PBS. The peptides dissolved in PBS were then added to 100 μl of the stock hRBCs suspended in PBS (final RBC concentration, 8% v/v). The samples were then incubated with agitation for 60 min at 37 °C, and then centrifuged for 10 min at 800×*g*. The absorbance of the supernatants was assessed at 414 nm; the controls for zero hemolysis (blank) and 100% hemolysis were comprised of hRBCs suspended in PBS and 1% Triton X-100, respectively. Each measurement was conducted in triplicate.

2.5. Cell line and culture

The human keratinocyte HaCaT cell line was obtained from Dr. NE. Fusenig (Heidelberg, Germany). Cells, cultured in 75 cm^2 plastic flasks, were grown in