The origin of cecropins; implications from synthetic peptides derived from ribosomal protein L1

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Abstract We recently showed that *Helicobacter pylori* grown on plates produce cecropin-like antibacterial peptides to which *H. pylori* is resistant. This antibacterial activity was traced to fragments from the N-terminus of ribosomal protein L1 (Pütsep et al., Nature, April 22, 1999). The evolutionary suggestion from this finding has now been extended by the synthesis of eight peptides with sequences taken from the N-terminus of ribosomal protein L1 (RpL1) of five different species. Two peptides of different length derived from *H. pylori* RpL1 showed a potent antibacterial activity, while a peptide with the sequence from *Escherichia coli* was 20 times less active. Like cecropins the *H. pylori* peptides were not cytolytic. We suggest that the cecropins have evolved from ribosomal protein L1 of an ancestral intracellular pathogen that developed to a symbiont ending as an organelle. When the R1 gene moved into the host nucleus, a duplication provided a copy from which today cecropins could have evolved.

Key words: *Helicobacter pylori*; Natural microflora; Cecropin; Evolution

1. Introduction

Gene-encoded antibacterial peptides constitute the central effector molecules of innate immunity and based on their 3D structures they have been grouped into three or five distinct classes [1,2]. One group have six cysteines, the α- and β-defensins forming flat β-sheets [3]. Defensins are widely distributed among animals and insects. Plants have similar molecules, but so far no such peptides are known from microorganisms. An other group of peptides are free of cysteines and they often form amphipathic α-helices such as the cecropins. The 3D structure of cecropin A was determined [4]. Numerous peptides have been designed, many of them based on cecropin sequences, in part with the aim of obtaining potential drug candidates [5–7].

Gene-encoded antibacterial peptides are made by animals and plants as well as by some bacteria. A group called lanthibiotics are posttranslationally undergoing several chemically interesting modifications [8]. However, a number of linear peptides with antibacterial activity such as lactococcin A are also known. Surprisingly, we recently found that *Helicobacter pylori* grown in vitro contains a significant antibacterial activity that could be traced to several cecropin-like peptides derived from the N-terminal part of ribosomal protein L1 [9]. Since this finding would provide a lead to the evolution of the cecropins, we report here the activity of several synthetic peptides corresponding to the N-terminal sequences of ribosomal proteins from five different species. Of those made so far, the *Helicobacter* peptides are most potent.

2. Materials and methods

2.1. Synthetic peptides and sequences for ribosomal L1 proteins

The peptides derived from the N-terminus of five different ribosomal proteins L1 were purchased from Neosystem (Strasbourg, France). The purity of the peptides was checked by the manufacturer using HPLC. All sequences for the ribosomal proteins L1 were obtained from GenBank. The length of peptides Hp(2–13), Hp(2–20), Hp(2–38), Hp(20–38), Ec(2–19), Tt(2–19), Pp(2–18) and Syn(2–20) are indicated by solid lines under the respective sequences in Fig. 1. Cecropins A and P1 as well as melittin were kindly synthesized some time ago by David Andreu and David Wade.

2.2. Bacterial strains and antibacterial assays

Escherichia coli K12 strain W3110 expresses a wild-type lipid A in contrast to its *msb*B mutant which is defective in the final acylation step of the lipid A part of its lipopolysaccharide (LPS), [10]. *E. coli* D21 and *Bacillus megaterium* strain Bm11 are streptomycin resistant mutants frequently used as assay organisms [11].

Antibacterial activity was determined using the inhibition zone method [11]. Indicator bacteria were suspended in 6 ml of LB agarose and casted in a 9 cm Petri dish. Small holes (diameter 3 mm) were punched in the thin bacteria agarose layer. For each peptide, a 2-fold dilution series of the peptide was applied to the holes and the plates were incubated overnight at 28°C. The diameters of the growth inhibition zones were used to calculate the lethal concentration (LC in μM) which is the lowest concentration of peptide that will inhibit bacterial growth. The LC values were calculated using the formula given in [11]. Small zones deviate from the linear relation and in these cases the LC values were calculated directly from the diameter using the alternative formula [11].

In the killing rate experiment, 10 μl aliquots were withdrawn at indicated time points, diluted to 100 μl and spread on LB plates for viable count determination. The concentration of the Hp(2–20) peptide was 3.5 μM.

3. Results

Fig. 1 shows the N-terminal sequences for ribosomal protein L1 from five different species and the sequence for cecropins A and B from *Hyalophora cecropia*. Residues 3–11 in the cecropins and in *H. pylori* L1 constitute a highly conserved box with six identical residues and three highly conserved residues (Fig. 1). The 3D structure of cecropin A shows that the molecule has two helices (residues 4–21 and 25–37) connected by a hinge composed of residues AGP [4]. The *Helicobacter* sequence can be plotted into two helices (residues 1–18 and 22–38). Fig. 2 shows that the first helix from *H. pylori* L1 and from cecropin can both be plotted as perfect or almost perfect amphipathic Edmundson wheels. In the wheel for the *E. coli* protein the A2 residue is interrupting the hydrophilic

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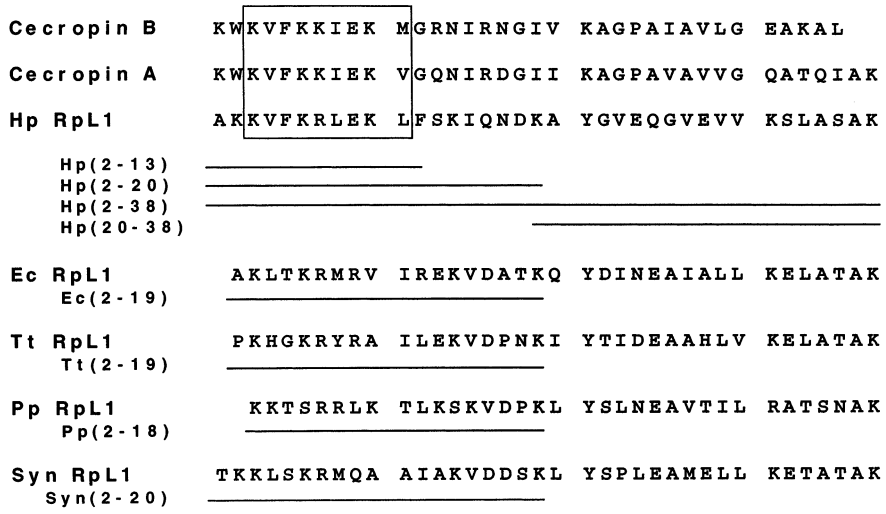

Fig. 1. Sequences for the N-terminal part of ribosomal protein L1 from five different species, *H. pylori* (Hp), *E. coli* (Ec), *T. termophilus* (Tt), *P. porpurea* chloroplast (Pp) and *Synechocystis* PCC 6803 (Syn). The length of the peptides synthesized are indicated by solid lines below the respective sequences. For comparison the top part shows the sequences for cecropins B and A from *H. cecropia*. The boxed in sequences show the most similar part of the *H. pylori* and the cecropin sequences.

side of the wheel, an effect that could be corrected by a first base substitution.

An important characteristic found when the first cecropins were isolated [12] was that they did not lyse chromium labelled animal cells, while the bee venom peptide melittin was highly lytic. Fig. 3 shows that neither the Hp(2–20) nor cecropin P1 lysed the intestinal cell line AGS while melittin gave 80% lysis. A prolonged incubation for 18 h gave the same result (data not shown).

We have synthesized eight N-terminal peptides (see Fig. 1), four with sequences derived from *H. pylori* L1 and four from four other species and tested them for antibacterial activity against *B. megaterium* and *E. coli* K12. One of the *H. pylori* peptides was devoid of activity, namely Hp(22–38) and this was the case also for the peptides derived from the N-terminal parts of the L1 proteins of *P. porpurea* chloroplast, *T. thermophilus* and *Synechocystis* strain PCC 6803. The antibacterial activities found against our common assay organisms *E. coli* D21 and *B. megaterium* Bm11 are given in Table 1. Clearly the most active peptide was Hp(2–38) containing both helices and thus conceptually being most similar to the cecropins. However, both of the shorter *H. pylori* peptides were active. The *E. coli* peptide showed a low but clearly detectable activity against *B. megaterium*. The presence of Medium E [13] gave a 20-fold increase in potency for the long *H. pylori* peptide, most likely an effect due to increased helix formation as previously documented for FALL-39 [14].

It should be stressed that all peptides were found to be inactive on *H. pylori*. Table 1 shows that in general the Gram-positive *B. megaterium* was more susceptible to the RpL1 derived peptides than was *E. coli*. However, the *E. coli* LPS mutant *msb*B (with only five fatty acid residues instead of normally six) was clearly more sensitive to the *H. pylori* peptides than was the wild-type. Interestingly, none of the cecropins detected this LPS difference.

As pointed out before an effector of innate immunity should be able to eliminate an invading organism in a time shorter than the doubling time of the bacterium [2]. We have therefore performed a rate experiment using the peptide Hp(2–20) and the bacterium *B. megaterium*. Fig. 4 shows that viable count dropped to zero after 8 min incubation at 37°C with a peptide concentration closed to the LC value.

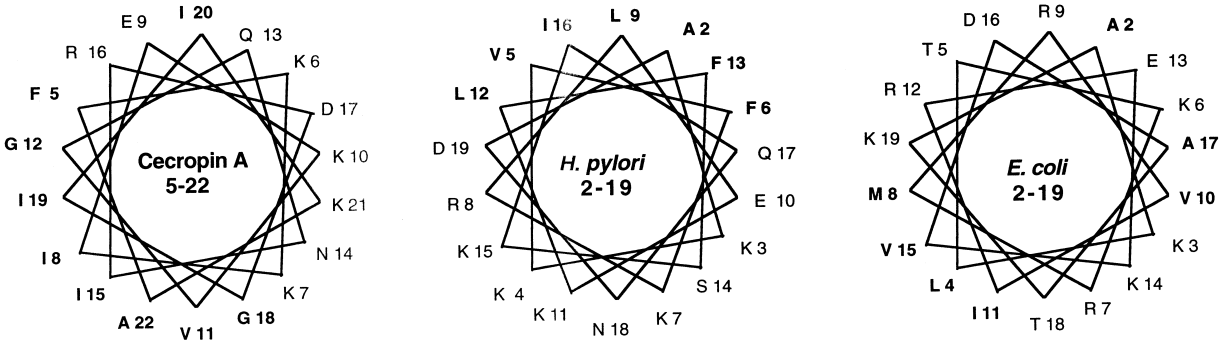

Fig. 2. Edmundson wheel diagram of cecropin A, *H. pylori* ribosomal protein L1 and *E. coli* ribosomal protein L1. The residue numbers are given in the center under the names. The *H. pylori* wheel is a perfect amphipathic α-helix with the upper part hydrophobic and the lower part hydrophilic.

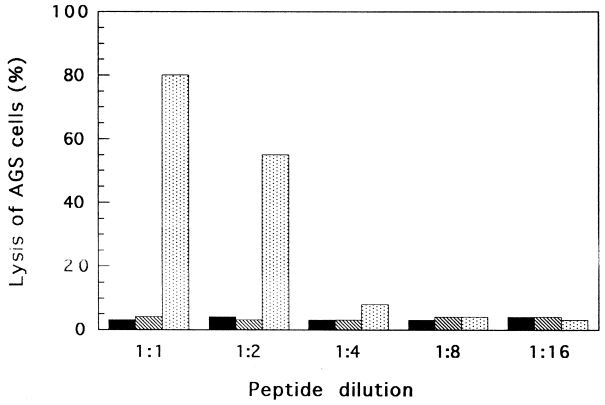

Fig. 3. Release of ^{51}chromium from the human intestinal cell line AGS after 2 h incubation at 37°C with decreasing amount of different peptides. Filled columns Hp(2–20), highest conc. 23 μM; striped columns cecropin P1 and dotted columns melittin, for both highest conc. 3 μM.

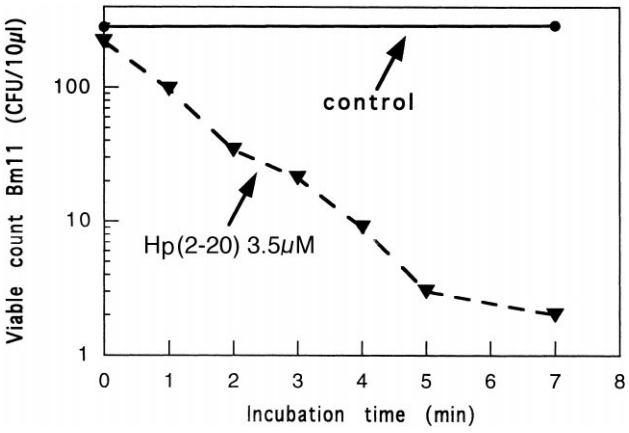

Fig. 4. Rate of killing of *B. megaterium* by the synthetic peptide Hp(2–20) at 37°C. The solvent was LB medium and the control was without any addition. The last sample taken at 8 min was sterile.

4. Discussion

Five years ago a global eradication of *H. pylori* was discussed [15]. The finding of cecropin-like peptides in extracts of *H. pylori* together with the known fact that the bacterium can undergo 'altruistic lysis' [16] opens the possibility that the organism is of importance for the natural balance of the human microflora [9]. Thus, it not clear that a universal eradication of *H. pylori* should be undertaken before more knowledge is accumulated about the dynamics of the normal human microflora.

The Hp RpL1 peptides and two cecropins were inactive against the strains of *H. pylori* tested (A5, MO19, P466, and CCUG17874) [9]. It has been suggested that cecropin A binds to the diphosphoryl lipid A moiety of LPS [17]. The lower amounts of phosphates in *H. pylori* lipid A [18] could be one way by which this organism is cecropin resistant. The *msb*B mutant, lacking one fatty acid residue in its LPS, was more sensitive to Hp(2–20) than was the wild-type (Table 1). This difference was not detected by the cecropins. Like the cecropins, the *Helicobacter* peptide HP(2–20) showed no lytic or toxic activity against the intestinal cell line AGS (Fig. 3).

It has been suggested that the first gene-encoded peptide antibiotics did evolve from a competitive need of slow-growing eukaryotic organisms to counteract fast-growing organisms in the same ecosystem [1]. Since bacterial cell surfaces in general, like DNA and RNA, are negatively charged, DNA and RNA binding proteins are obvious candidates for the origin of antibacterial peptides. The fish antibacterial peptide parasin I was shown to be processed from histone H2A [19]. Thus, in itself it could be expected that ribosomal proteins are candidates for the evolution of antibacterial peptides. For *E. coli* RpL1, RNAse protection assays and genetic evidence show that the charged N-terminus is involved in RNA binding [20]. Also the *E. coli* RpL1 peptide Ec(2–19) had activity, but only against a Gram-positive bacteria and it was around five times less active than the *H. pylori* peptide Hp(2–20) (Table 1). The fact that the longer *Helicobacter* peptide Hp(2–38) has two potential helical regions and a much higher activity than Hp(2–20) does emphasize the similarity with the moth cecropins.

The L1 peptides derived from the thermophilic bacterium *T. thermophilus* Tht(2–19), the alga *Synechocystis* PCC 6803 Syn(2–20) and plant *P. porpurea* chloroplast Pp(2–18) were all devoid of activity (data not shown). Thus, these findings do not provide any evidence about the evolution of the cecropins from the L1 proteins of these three organisms. However, the sequences derived from two Gram-negative bacteria (*H. pylori* and *E. coli*) suggest that the cecropins could have evolved from an early RpL1 gene in a particular prokaryote. This ancestral bacterium may have been *H. pylori* like and changed from being an intracellular parasite of an ancient eukaryotic organism to become a symbiont and finally ending up as an organelle of the host. Since many organelle host genes today are located in the nucleus, one can suggest that a duplicated copy of the L1 gene served as the original genetic material from which today cecropins have evolved.

Table 1
Antibacterial activity of four L1 derived peptides and two cecropins

Strain peptide	D21	W3110	*msb*B	Bm11	Bm11+MedE
Hp(2–13)	200	>570	30	5	8
Hp(2–20)	20	35	8	5	3
Hp(2–38)	5	5	3	8	0.4
Ec(2–19)	>260	>260	>260	20	19
Cecropin A	0.4	0.2	0.1	0.6	0.7
Cecropin P1	0.2	0.1	0.2	4	3

Activities are given as lethal concentrations (LC values in μM) [11]. Strains D21 and W3110 are derivatives of *E. coli* K12 and the latter is the parental strain for the *msb*B mutant. Bm11 is a streptomycin resistant mutant of *B. megaterium*. Other details are given in Section 2. Medium E (MedE) is used because it can promote helix formation [14].

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