Antimicrobial Activity of Rabbit CAP18-Derived Peptides

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Received 28 July 1993/Returned for modification 30 August 1993/Accepted 20 September 1993

A cationic antimicrobial protein of 18 kDa (CAP18) was originally isolated from rabbit granulocytes by using as an assay the agglutination of Re-lipopolysaccharide-coated erythrocytes. The C-terminal 37-amino-acid fragment of CAP18 (CAP18106-142) make up the lipopolysaccharide-binding domain. Synthetic CAP18106-142 has broad antimicrobial activity against both gram-positive (50% inhibitory concentration, 130 to 200 nM) and gram-negative (50% inhibitory concentration, 20 to 100 nM) bacteria. Susceptible strains include Staphylococcus aureus, Streptococcus pneumoniae, and Salmonella typhimurium. Antimicrobial activity is highly dependent on peptide structure. Although a 32-amino-acid peptide resulting from the truncation of 5 amino acids from the N terminus of CAP18106-142 is highly active, other fragments of CAP18106-142, including CAP18106-142 with a truncated N terminus, do not exhibit antimicrobial activity. Unlike previously characterized antimicrobial peptides derived from granulocyte proteins, CAP18106-142 is active in serum. CAP18106-142 or a derivative peptide may have therapeutic potential for bacterial sepsis.

Polymorphonuclear neutrophils contain a number of proteins and peptides that have antimicrobial activities. Among the best characterized are bactericidal- and permeability-increasing protein (BPI) (3, 13), the defensins (9), and azurocidin (a cationic antimicrobial protein of 37 kDa [CAP37]) (11). We recently reported the isolation and characterization of a novel lipopolysaccharide (LPS)-binding protein called CAP18 (4, 5). CAP18 was identified and purified on the basis of its ability to agglutinate erythrocytes coated with LPS, especially Re LPS and lipid A (6). Early studies demonstrated that semipurified preparations of CAP had antibacterial activity (14). Cloning and sequencing of the cDNA of rabbit CAP18 led to the discovery of a C-terminal 37-amino-acid fragment, designated CAP18106-142, with LPS-binding activity (8). Here we demonstrate that CAP18106-142 and a related, slightly shorter fragment have potent antimicrobial activities against both gram-negative and gram-positive bacteria. This activity is highly dependent on structure, because other truncated segments of the 37-amino-acid fragment are inactive. Because antibacterial activity is maintained in the presence of serum, CAP18106-142 or a derivative thereof may have therapeutic potential.

MATERIALS AND METHODS

Antibacterial activity. (i) Bacterial strains. Salmonella typhimurium LT2 (smooth), Salmonella minnesota R595 (Re mutant), Escherichia coli O9:K39 (K+ and K- strains), E. coli O111:B4, Streptococcus pneumoniae, Streptococcus pyogenes, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus (methylcin susceptible and methicillin resistant), and Candida albicans were used. The last five strains were clinical isolates.

(ii) Bacterial assay. All strains were grown in Tryptose Broth (Eiken Co., Tokyo, Japan). S. typhimurium LT2 (smooth) S. minnesota R595 (Re), E. coli O9:K39 (K+ and K-), E. coli O111:B4, and K. pneumoniae were plated on nutrient agar (Eiken Co.). P. aeruginosa and C. albicans were plated on NAC agar and guanofracion-Sabouraud agar plates, respectively. Bacterial cultures were collected at the logarithmic phase of growth, washed twice with phosphate-buffered saline (PBS; pH 7.2), and adjusted to a final concentration of 5 x 10^9 to 1 x 10^10 cells per ml. To 450 μl of bacterial suspension, 50 μl of peptide was added and the mixture was incubated at 37°C for 1 h; 100 μl of the reaction mixture was then plated onto the agar plate. After 24 h of incubation at 37°C, the number of CFU was counted. As a control experiment, PBS was added to the bacterial suspension and the mixture was incubated for 1 h, plated on agar, and cultured. For some experiments the percentage of the control CFU was determined.

3H-thymidine incorporation bacterial proliferation assay. Bacteria were grown in Trypticase broth overnight. On the following day, bacteria were suspended at 10^7/ml in RPMI 1640 medium with 10% fetal calf serum (FCS). The assay was set up in a 96-well plate containing 50 μl of bacteria plus 50 μl of peptides, and the mixture was incubated at 37°C for 1 h; then, 1 μCi of [3H]thymidine (Amersham) was added to each well, and the plates containing the bacteria were incubated overnight. The assay was terminated by the addition of 10% trichloroacetic acid, and cells were harvested and counted on a Matrix 96 Packard beta counter.

Statistical analysis. Results are expressed as means ± standard deviations for at least three or four plates. Student’s t test for unpaired data was used to determine statistical significance. A two-tailed P value of <0.05 was considered significant. Fifty percent inhibitory concentrations (IC50s) were determined by least-squares linear regression.

Preparation of peptides. Rabbit CAP18 is a 142-amino-acid protein. Peptide fragments of CAP18 were numbered accordingly. Peptide synthesis was carried out on an Applied Biosystems model 430A or model 431A peptide synthesizer. High-pressure liquid chromatography (HPLC) was performed on an LDC apparatus equipped with two Constametric pumps, a Gradient Master solvent programmer and mixer, and a Spectramon III variable-wavelength UV detector. Fast atom bombardment mass spectra were obtained on a VG 70E-HF mass spectrometer. Final purifica-
tion was carried out by preparative HPLC. The peptides were applied to the column in a minimum volume of either 10 to 20% acetyl hydroxide or 0.1% trifluoroacetic acid (TFA). Gradient elutions were performed by using linear gradients of buffer A (0.1% TFA and H2O) and buffer B (0.1% TFA and CH3CN) at a flow rate of 8.0 ml/min with UV detection at 220 nm. Fractions were collected at 1.5- to 2.5-min intervals and were inspected by analytical HPLC in the reversed-phase mode as stated above. Fractions judged to be of high purity were pooled and lyophilized. The final products were characterized by analytical HPLC and amino acid analysis. The purified peptides were also subjected to fast atom bombardment mass spectrometry and yielded the expected parent M+H ions within acceptable limits (±1 mass unit).

Erythrocyte agglutination assay. One milliliter of 1% erythrocytes (human type O, C3H/HeN mouse, or sheep erythrocytes) were sensitized and mixed with 0.2 ml of Re LPS solution (100 μg/ml), and the mixture was incubated at 37°C for 30 min; this was followed by washing with PBS. Fifty microliters of a 1.0% suspension of sensitized erythrocytes was mixed with 50 μl of a twofold serial dilution of CAP or CAP peptides in a U-bottom microtiter plate, and the mixture was incubated at 37°C for 1 h. The activity of CAP was expressed as the minimum agglutinating concentration.

Assay of reactive nitrogen intermediates. The murine macrophage cell line RAW 264.7 (obtained from the American Type Culture Collection) was used to produce reactive nitrogen intermediates. Cells were cultured at 10^6/ml in RPMI 1640 medium–2.5% FCS in 24-well plates in the presence or absence of different concentrations of LPS. After 24 h of incubation at 37°C, the cell-free supernatant was collected and tested for the presence of reactive nitrogen intermediates. Accumulation of nitrite in the medium was measured by a colorimetric assay on the basis of the Griess reaction with sodium nitrite standards. Sample (50 μl) was mixed with 50 μl of Griess reagent (1% sulfanilamide, 0.1% naphthalene diamine dihydrochloride, 2.5% H3PO4), and after 10 min at room temperature the A570 was read.

**RESULTS**

LPS binding by CAP18106-142 peptides. In a previous report (7) a large series of CAP18106-142-derived peptides was studied for LPS binding and neutralization of LPS-induced nitric oxide release from murine macrophages. From that initial work, four peptides were selected for in-depth studies: native CAP18106-142, a more active peptide with a truncation at the C-terminal end of CAP18106-142 (peptide CAP18106-137), and two inactive peptides, CAP18106-114 and CAP18117-142. Table 1 shows the sequences and the LPS-binding activities of the four peptides for their antimicrobial activities. Table 1 also shows the high degree of correlation of LPS binding with inhibition of LPS-induced production of nitric oxide.

CAP18106-142 peptides inhibit growth of diverse strains of gram-negative bacteria. Because CAP18106-142 was originally shown to bind and cause agglutination of erythrocytes coated with rough mutant LPS, antimicrobial activity against the rough mutant strain S. minnesota R595 was initially evaluated. Figure 1A presents the dose-responses of the four peptides on S. minnesota R595 CFU. Non-LPS-binding peptides CAP18106-114 and CAP18117-142 were not active, whereas the LPS-binding peptides CAP18106-142 and CAP18106-137 had significant antibacterial activities (IC50 <100 ng/ml; 20 nM).

Next, the activity against a smooth enterobacterial strain, S. typhimurium, was tested (Fig. 1B). The LPS-nonbinding peptides CAP18106-114 and CAP18117-142 were not active, whereas the LPS-binding peptides CAP18106-142 and CAP18106-137 had significant antibacterial activities (IC50 220 to 280 nM). Figure 1C demonstrates that CAP18106-142 had significant activity against two other clinical strains of bacteria, P. aeruginosa (IC50 <250 ng/ml; 40 nM) and K. pneumoniae (IC50 <440 ng/ml; 70 nM). Similar results were obtained when peptide CAP18106-137 was tested against P. aeruginosa (IC50 <250 ng/ml; 50 nM) and K. pneumoniae (IC50 <540 ng/ml; 100 nM) (data not shown).

Two other control experiments were carried out with the LPS-nonbinding peptides. In the first case (Fig. 2A), pep-
FIG. 1. Antibacterial activity of CAP18<sub>106-142</sub> synthetic peptides against gram-negative bacteria. (A) Rough mutant strain S. minnesota Re. (B) Smooth enterobacterial strain S. typhimurium LT2. The LPS-nonbinding peptides, CAP18<sub>106-114</sub> (open squares) and CAP18<sub>117-142</sub> (closed squares) did not inhibit bacterial growth. The LPS-binding peptides CAP18<sub>106-142</sub> (open circles) and CAP18<sub>106-137</sub> (closed circles) inhibited bacterial growth. (C) The results obtained with peptide CAP18<sub>106-142</sub> against clinical isolates P. aeruginosa (open squares) and K. pneumoniae (closed circles).

FIG. 2. Specificity of antibacterial activity of CAP18<sub>106-142</sub> synthetic peptides against S. typhimurium LT2. (A) The LPS-nonbinding peptides CAP18<sub>106-114</sub> and CAP18<sub>117-142</sub> did not exhibit activity alone or in various combinations even at high concentrations. Bacterial cell suspensions were incubated with peptides at 37°C for 1 h. #32-1, CAP18<sub>106-114</sub> #50-2, CAP18<sub>117-142</sub>. (B) The LPS-nonbinding peptide CAP18<sub>106-114</sub> did not inhibit the activity of the antibacterial peptide CAP18<sub>106-142</sub>. Bacterial cell suspensions were incubated with peptide CAP18<sub>106-114</sub> at 37°C for 30 min, and then peptide CAP18<sub>106-114</sub> was added and the mixture was incubated for 1 h. #32-1, CAP18<sub>106-114</sub> #197-1, CAP18<sub>106-142</sub>.

tides CAP18<sub>106-114</sub> and CAP18<sub>117-142</sub> were added at high concentrations alone and in various combinations to determine their effects on the CFU of S. typhimurium. No effect was observed. In the second case, S. typhimurium was pretreated with peptide CAP18<sub>106-114</sub> and was subsequently exposed to active peptide CAP18<sub>106-142</sub> (Fig. 2B). In this case, no inhibition or augmentation of bacterial killing by CAP18<sub>106-142</sub> was observed. Results of these experiments, in conjunction with the results of those carried out as described above, demonstrate that for CAP18<sub>106-142</sub> to have an inhibitory effect, it must meet specific structural requirements.

To corroborate the antibacterial activities shown by the CFU assays, inhibition of proliferation of bacteria was tested directly by a [<sup>3</sup>H]thymidine incorporation assay. The assay also addressed any artifacts resulting from clumping of the test strains mediated by CAP18<sub>106-142</sub> peptides that would lead to false interpretation of a CFU type of assay. The results shown in Table 2 demonstrate that CAP18<sub>106-142</sub> inhibits the proliferation of E. coli, K. pneumoniae, and two strains of Pseudomonas. In summary, although there was some strain-to-strain variation in the IC<sub>50</sub>s, all of the gram-
negative strains tested in two different types of assays were susceptible to these antimicrobial peptides.

In further studies, the susceptibilities of encapsulated strains were examined. Figure 3 compares the activity of peptide CAP18106142 against encapsulated (K⁺) and nonencapsulated (K⁻) E. coli O9:K39. The K⁻ strain was less susceptible to the peptide (IC₅₀, 700 ng/ml; 140 nM) than the K⁺ strain (IC₅₀, 140 ng/ml; 30 nM).

Finally, the effect of serum on killing was studied, because other antimicrobial peptides require nonphysiological conditions (e.g., hypotonic buffers and acidic pH [5.0 to 5.5]) to exhibit activity. The addition of 10% FCS to the proliferation assay performed in RPMI 1640 medium resulted in only a modest decrease in antibacterial activity (13 to 17%; data not shown).

**TABLE 2. Bacterial proliferation inhibition by peptide CAP18106-137**

<table>
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<tr>
<th>Group and strain</th>
<th>% Inhibition at the following concn (µg/ml) of CAP18106-137</th>
<th>5.0</th>
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<th>0.6</th>
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<td>99</td>
<td>88</td>
<td>81</td>
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<td>K. pneumoniae</td>
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<td>4</td>
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</tr>
<tr>
<td>P. aeruginosa</td>
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<td>96</td>
<td>82</td>
<td>75</td>
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</tr>
<tr>
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<td>99</td>
<td>80</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>Gram-positive bacteria</td>
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<td>31</td>
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<td>72</td>
<td>57</td>
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</table>

* Percent inhibition of proliferation as determined by [³H]thymidine incorporation assay; results are representative of three experiments.

FIG. 3. Antibacterial activity of CAP18106-142 synthetic peptide. The E. coli O9:K39 K⁺ strain (open squares) was more susceptible than the K⁻ strain (closed squares).

FIG. 4. Antibacterial activity of CAP18106-142 synthetic peptide against gram-positive bacteria. The dose-response of antimicrobial activity of peptide CAP18106-142 against methicillin-susceptible (MSSA) and methicillin-resistant (MRSA) S. aureus and E. coli (in CFU) is shown as a percentage of that for the PBS-treated control (see text).

**DISCUSSION**

In the present study we defined the potent antimicrobial activities of peptides derived from the rabbit granulocyte protein CAP18. The cloning of rabbit CAP18 revealed that this molecule comprises two domains (8). The N-terminal domain is highly homologous to porcine cathelin originally purified as a cysteine protease inhibitor (12). Purified human CAP18 also demonstrates significant cysteine protease activity (17). The C-terminal 37-amino-acid domain (CAP18106-142) of CAP18 was identified as the LPS-binding domain when HPLC fractions were tested for their capacity to inhibit LPS-induced nitrogen radical production (5). Previously, a large series of CAP18106-142-derived peptides was studied for LPS binding and neutralization of LPS-induced nitric oxide release from murine macrophages (7). That work demonstrated that truncation of the N terminus of the protein resulted in loss of LPS-binding activity. The two best LPS-binding peptides, CAP18106-142 and CAP18106-137 and...
two fragments without LPS-inhibitory activity, were chosen for the present study (Table 1). Peptides CAP18~96-142 and CAP18~96-137 had potent (submicromolar) antibacterial activities against all gram-negative strains tested and against many gram-positive strains. Furthermore, activity was demonstrated in the presence of serum at neutral pH in a physiological medium. No activity against C. albicans or two *Mycobacterium* species was demonstrated.

The outer membrane of gram-negative bacteria provides an effective permeability barrier against external noxious agents, including antibiotics. Numerous studies have shown that antibacterial agents such as polycations and chelators weaken the molecular interactions of the LPS with the outer membrane (15). Polycations can, under certain conditions, bind to the anionic sites of LPS. Many molecules disorganize and cross the outer membrane and render it leaky to drugs that normally permeate the intact outer membrane very poorly. Such polycations include polyymyxins and their derivatives, protamine, polymers of basic amino acids, compound 48/80, insect cecropins, reptilian magainins, various cationic leukocyte peptides (defensins, bactenecins, BPI, and others), and aminoglycosides (15). From the results of the present study and previous work, it is clear that the cationic nature of these agents is not the sole determinant required for the permeabilizing activity.

The activity of CAP18 can be contrasted with that of BPI, which was originally shown to have antibacterial activity against a variety of gram-negative bacteria, with no activity against gram-positive bacteria or fungi (13). BPI demonstrates high-affinity binding to LPS (3) and can apparently inhibit the activity of LPS in vitro (10). Both the aminoterminus fragment (rBPI~123) and the holoprotein (BPI~33) inhibit encapsulated *E. coli*. While both BPI~55 and rBPI~33 inhibited the growth of a rough mutant strain of *Proteus mirabilis*, only rBPI~33 inhibited growth of the wild-type smooth organism. Neither rBPI~33 nor holoprotein BPI~55 inhibit the growth of gram-positive *S. aureus* (16).

Three other families of granulocyte proteins exhibit LPS-binding and antimicrobial activities. These include the 13-amino-acid C-terminal peptide of bovine indolicon (2), the 30- to 35-amino-acid family of defensins (9), and azurocidin (CAP37) (11). The indolicidin peptide and the defensins only inhibit the growth of gram-positive and gram-negative bacteria in hypotonic medium, thus distinguishing them from CAP18~96-142. A recent report (11) described peptides derived from CAP37 which were active at concentrations approximately 2 to 3 log units higher than those derived from CAP18.

Many questions remain regarding the mechanism of CAP18~96-142 peptide-mediated antimicrobial activity. Limited structure-activity studies of CAP18~96-142 peptides demonstrate a 100% correlation between binding of LPS and antimicrobial activity. Thus, the LPS-binding peptides CAP18~96-142 and CAP18~96-137 exhibit broad antimicrobial activity, whereas LPS-nonbinding peptides are inactive. This indirect evidence suggests that LPS binding participates in the antibacterial activity against gram-negative bacteria. Previously, we showed that truncation of more than five amino acids from the N terminus of CAP18~96-142 inactivated the protein (7). Peptide CAP18~96-137, a five-amino-acid truncation of the C terminus of CAP18~96-142, is as potent as or has greater activity than the parental peptide CAP18~96-142. Other truncations of the N terminus exhibit less activity than CAP18~96-137, although these have not been tested in detail.

Future work will address several important questions. For example, what target structures on the surfaces of gram-positive bacteria are recognized by CAP18~96-142, and how does CAP18~96-142 mediate cytotoxicity to these microorganisms? Although in the present study we did not directly address these questions, the antimicrobial effect is highly selective because no activity was observed against *Mycobacterium* spp. or *C. albicans*. We hypothesize that part of the growth-inhibitory activity of CAP proteins is mediated by binding to cell wall components bearing a negative charge. The fact that the cell wall of *C. albicans* comprises mannan and other neutral sugars may partially explain the resistance of fungi to CAP proteins. Like gram-negative bacteria, the cell walls of gram-positive bacteria contain negatively charged molecules such as lipoteichoic acids. Interestingly, a common protein binds LPS and lipoteichoic acids in serum (1). Future studies will examine the possibility that CAP18~96-142 mediates its effects against gram-positive organisms via binding to lipoteichoic acid.

ACKNOWLEDGMENTS

This work was supported in part by NIH grant RO1-GM47720 and by a grant-in-aid (04670250) for Scientific Research from the Ministry of Education of Japan.

REFERENCES


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