J. Biochem. 131, 277–281 (2002)

Participation of Two N-Terminal Residues in LPS-Neutralizing Activity of Sarcotoxin IA[1]

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Received October 23, 2001; accepted December 12, 2001

Sarcotoxin IA is a cecropin-type antibacterial peptide of flesh fly. Using a mutant sarcotoxin IA lacking two N-terminal residues, we demonstrated that these residues are indispensable for its antibacterial activity against *Escherichia coli* and LPS-binding. Contrary to the native sarcotoxin IA, the mutant sarcotoxin IA could not neutralize various biological activities of LPS. It was suggested that sarcotoxin IA firmly binds to the lipid A core of LPS *via* these two N-terminal residues and forms a stable binding complex that exhibits no appreciable biological activity like native LPS.

Key words: antibacterial peptide, cecropin A, insect immunity, LPS, sarcotoxin IA.

The sarcotoxin I family comprises a group of cecropin-type antibacterial peptides of *Sarcophaga peregrina* (flesh fly), each consisting of 39 amino acid residues (*1*). At least five sarcotoxin I congeners are present in this insect (*1, 2*). Among them, the mode of action of sarcotoxin IA has been extensively studied. Sarcotoxin IA exhibits potent bactericidal activity against Gram-negative bacteria (*3, 4*). Sarcotoxin IA is expected to be amphiphilic and to interact readily with bacterial membrane (*5*). In fact, we found that sarcotoxin IA disrupts the electrochemical potential of bacterial membranes, resulting in cessation of ATP synthesis and amino acid transport in *Escherichia coli*. Moreover, on treatment with sarcotoxin IA, *E. coli* becomes extremely susceptible to detergents such as SDS (*3, 4*). The N-terminal half of this molecule is rich in positively charged amino acids and is hydrophilic, whereas the C-terminal half is hydrophobic (*1*). Thus, it was proposed that the N-terminal half of this molecule interacts with acidic phospholipids and that the C-terminal half penetrates into the bacterial membranes, causing membrane perturbation (*5*).

Recently, bacterial lipopolysaccharide (LPS) was found to block the antibacterial activity of cecropin A, suggesting an interaction between cecropin A and LPS (*6*). On the other hand, it has been pointed out that the Trp residue at position 2 of cecropin A is crucial for its antibacterial activity. Namely, a significant loss of antibacterial activity was detected when the two N-terminal residues of cecropin A (Lys^1 and Trp^2) were removed. Furthermore, when Glu was substituted for Trp at position 2, the antibacterial activity of the resulting mutant cecropin A was greatly reduced, being 100-times lower than that of authentic cecropin A depending upon the target bacterium (*7*).

Taking these facts together, we assumed that sarcotoxin IA might bind directly to LPS on the surface of Gram-negative bacteria, and that the Trp at position 2 of sarcotoxin IA participates in its binding to LPS. To prove this, we compared the biological activities of sarcotoxin IA and a mutant sarcotoxin IA lacking the two N-terminal residues (Gly^1 and Trp^2), with special reference to their interaction with LPS. We found that the two N-terminal residues are indispensable for sarcotoxin IA to interact with LPS. Removal of these two residues from sarcotoxin IA caused losses of both antibacterial activity and LPS-binding activity, but its ability to disrupt liposomes consisting of acidic phospholipids did not change appreciably regardless of the presence or absence of these two residues.

MATERIALS AND METHODS

Sarcotoxin IA and Its Derivative—Sarcotoxin IA and derivative of it were chemically synthesized at Peptide Institute (Osaka). In this study, we defined sarcotoxin IA that lacked the two N-terminal residues as mutant sarcotoxin IA. The peptide concentration was measured by the method of Lowry *et al.* (*8*).

Determination of Antibacterial Activity—Antibacterial activity of a test sample was assayed colorimetrically essentially as described by Yajko *et al.* (*9*). Briefly, *E. coli* W3110 (10^5 cells) was incubated with native or mutant sarcotoxin IA (5 nM to 50 μM) for 4 h at 37°C in the presence of an Alamar Blue solution (Biosource Inc.). The bacterial growth was determined spectrophotometrically according to the manufacture's manual.

Detection of Binding of LPS and Sarcotoxin IA—The bottom of each well of a vinyl chloride culture plate (Sumitomo Bakelite, Tokyo) was treated with 50 μM native or mutant sarcotoxin IA dissolved in PBS (8 mM phosphate buffer containing 130 mM NaCl and 3 mM KCl) overnight, and then the plate was washed three times with PBS containing 0.05% Triton X-100 (ICN Pharmaceutical). Coating of the wells with peptides were confirmed by immunofluorescence with antibodies against sarcotoxin IA. Fluorescein isothiocyanate (FITC)–labeled *E. coli* 026:B6 LPS (FITC-LPS, SIGMA) (50 μl, 50 μg/ml) was added to each well, followed by incubation for 20 min at 37°C. Under these condi-

[1] This work was supported by Core Research for Evolutional Science and Technology (CREST) of Japan Science and Technology Corporation (JST).

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tions, 1.3 μg/well of FITC-labeled LPS was bound to native sarcotoxin IA. The affinity of LPS to sarcotoxin IA was assessed by measuring replacement of bound FITC-labeled LPS with non-labeled LPS added to the reaction mixture. Bound FITC-LPS was detected by measuring the fluorescence of FITC.

Preparation of Liposomes Entrapping Glucose and Measurement of Glucose Released from Them—This was performed essentially as described previously (*10*). Liposomes with entrapped glucose were prepared from commercially available phosphatidylcholine (PC) and lipid A (*E. coli* F-583, SIGMA), when necessary, by the method of Kinsky *et al.* (*11*). Briefly, a mixture of PC and lipid A was suspended in 10 mM phosphate buffer (pH 7.0) containing 0.3 M glucose and 0.13 M NaCl, and then the suspension was sonicated. The resulting glucose-entrapped liposomes were washed three times by centrifugation with 20 mM phosphate buffer (pH 7.0) containing 0.3 M NaCl. The amount of PC in the liposomes was determined with a phospholipid-testwako kit (Wako Pure Chemicals Industries, Osaka). To examine the sensitivity of liposomes to peptides, various concentrations of the peptides dissolved in 10 μl of the same buffer were added to 10 μl of the liposome suspension. The mixtures were incubated at room temperature for 1 h and then liposomes were centrifuged at 5,800 ×*g* for 5 min. The amount of glucose released in the supernatant was determined with a Glucose-B test kit (Wako Pure Chemicals Industries, Osaka).

Binding of LPS to J774.1 Cells—A murine macrophage-like cell line, J774.1, was obtained from the Riken Cell Bank (Tsukuba), and the cells were cultured in RPMI 1640 medium supplement with 10% fetal calf serum at 37°C. FITC-LPS (1 μg) was added to 2×10^6 cells with various concentrations of native or mutant sarcotoxin IA, followed by incubation at 37°C for 1 h. The cell suspension was centrifuged at 150 ×*g* for 5 min. The precipitated cells were suspended in ice-cold phosphate buffer, and then the fluorescence of LPS bound to the cells was detected with an EPICS Elite ESP flow cytometer (Beckman Coulter) at the wavelength of 520 nm.

Assaying of TNF-α Produced by LPS-Stimulated J774.1 Cells—J774.1 cells were plated at a density of 2×10^6 cells/well and LPS was added to each well to a final concentration of 250 ng/ml with native or mutant sarcotoxin IA at concentrations ranging from 100 nM to 10 μM. The cells were incubated at 37°C for 6 h and then the amount of TNF-α produced in the medium was determined with an ELISA kit (R&D Systems).

Northern Blot Hybridization—Third instar larvae of *Sarcophaga* were injected with 5 μl of insect saline containing increasing amount of native or mutant sarcotoxin IA with 1 μg/ml of LPS. The larvae were kept under wet conditions for 9 h at 27°C, frozen on dry ice, and then stored at –80°C. Total RNA were extracted from the frozen larvae and heated at 60°C for 15 min in morpholinopropanesulfonic acid/sodium acetate buffer (pH 7.0) containing 50% formamide and 2.2 M formaldehyde, and then subjected to Northern blotting. The ^{32}P-labeled DNA probes used were 758 and 127 bp PCR fragments of the coding regions of the *Sarcophaga* lectin gene (*12*) and sarcotoxin IB gene (*2*), respectively. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA was detected as an internal reference.

RESULTS

Antibacterial Activity of Mutant Sarcotoxin IA—We synthesized a mutant sarcotoxin IA that lacks the two N-terminal residues, Gly and Trp, and compared its antibacterial activity with that of native sarcotoxin IA using *E. coli* as a target bacterium. We assessed bacterial growth by means of the Alamar Blue assay. As shown in Fig. 1, bacterial growth was almost completely inhibited in the presence of 1 μM sarcotoxin IA, but no appreciable antibacterial activity was detected with mutant sarcotoxin IA even at the concentration of 50 μM. These results indicated that the two N-terminal residues of sarcotoxin IA were indispensable for its antibacterial activity against *E. coli*. Similar results have been reported with cecropin A that lacked its two N-terminal residues (*7*).

Two N-Terminal Residues of Sarcotoxin IA Are Essential for Its Binding to LPS—De Luca *et al.* reported binding between cecropin A and LPS *in vitro*, suggesting the binding of cecropin A to Gram-negative bacteria *via* their surface LPS (*6*). As the mutant sarcotoxin IA lacking the two N-terminal residues showed no appreciable antibacterial activity, we examined if it is able to interact with LPS. For this, we coated the bottoms of the wells of a vinyl chloride

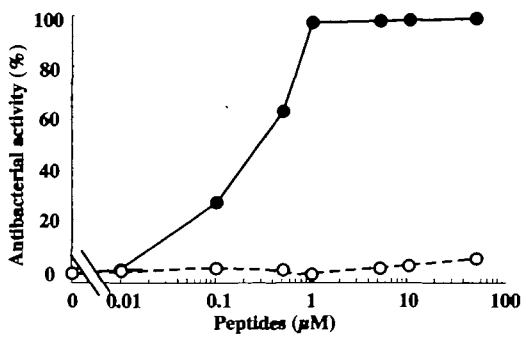

Fig. 1. **Antibacterial activity of mutant sarcotoxin IA against *E. coli*.** Antibacterial activity was examined by means of the Alamar Blue assay. The antibacterial activity as a percentage was plotted against the concentration of sarcotoxin IA used. ●, native sarcotoxin IA; ○, mutant sarcotoxin IA. Means of duplicate measurements were shown.

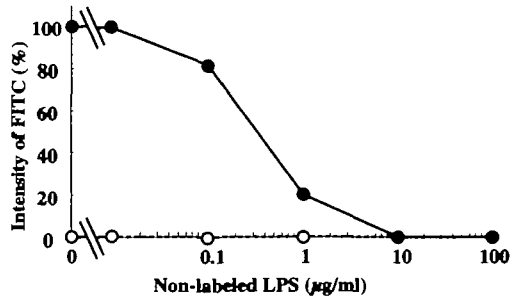

Fig. 2. **Binding of sarcotosin IA to LPS.** Peptide-coated culture plates were treated with FITC-LPS for 20 min at 37°C. Then increasing amounts of non-labeled LPS were added and incubated for 2 h at 4°C. The percentage of the fluorescence bound to the bottom of a well was plotted against the concentration of non-labeled LPS added to the well. Binding of FITC-LPS without a competitor was taken as 100%. ●, native sarcotoxin IA; ○, mutant sarcotoxin IA. Means of duplicate measurements were shown.

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culture plate with sarcotoxin IA and then assessed the binding of FITC-LPS to sarcotoxin IA by measuring the fluorescence of FITC attached to the bottom of the wells. Using an indirect immunofluorescence technique, we confirmed that the bottoms of the wells could be coated with both native and mutant sarcotoxin IA in the same way.

As shown in Fig. 2, no appreciable binding of FITC-LPS to mutant sarcotoxin IA was detected under the conditions where it bound significantly to intact sarcotoxin IA. The bound LPS was progressively replaced with non-labeled LPS when increasing amounts of non-labeled LPS were added to the reaction mixture. These results clearly indicate that sarcotoxin IA binds to bacterial LPS, and that the two N-terminal residues, Gly and Trp, are essential for its binding. These facts indicate that the binding between sarcotoxin IA and LPS is not simply due to their ionic interaction, because removal of two N-terminal residues should not affect the overall positive charge of sarcotoxin IA. Then the question arose as to how sarcotoxin IA interacts with LPS.

Previously, we demonstrated that sarcotoxin IA interacts with liposomes containing acidic phospholipids and made entrapped glucose permeable to lipid membranes, but its interaction with liposomes constructed from PC, a neutral phospholipid, was very weak (*10*). These results suggested that sarcotoxin IA only interacted with acidic phospholipids. To examine the involvement of LPS in the toxicity of sarcotoxin IA to Gram-negative bacteria, we constructed glucose-entrapped liposomes consisting of PC and increasing amounts of lipid A, and examined whether or not the liposomes became susceptible to sarcotoxin IA in a lipid A–dependent manner. Lipid A is the principal core structure of LPS and can be used as a model of LPS. As LPS has a complex structure, it was difficult to control the integration of LPS into liposomes quantitatively.

As is evident from Fig. 3, liposomes became sensitive to sarcotoxin IA with increases in the content of lipid A, and entrapped glucose was released from the liposomes at sarcotoxin IA concentrations above 5 μM. As the liposomes formed without lipid A were not disrupted even in the presence of 100 μM sarcotoxin IA, sarcotoxin IA does not seem to interact directly with PC. Contrary to native sarcotoxin IA, no appreciable glucose release was detected with mutant sarcotoxin IA irrespective of the lipid A content in the liposomes.

From these results, we concluded that sarcotoxin IA directly binds to lipid A and that the bound sarcotoxin IA interacts with phospholipid membranes, resulting in leakage of entrapped glucose. As expected, mutant sarcotoxin IA seemed to have completely lost the ability to bind to lipid A. Thus, we propose that sarcotoxin IA binds to the lipid A core of LPS *via* its two N-terminal residues. A similar situation may be expected when sarcotoxin IA interacts with intact Gram-negative bacteria.

LPS-Neutralizing Activity of Sarcotoxin IA—As it became evident that sarcotoxin IA binds to lipid A *via* its two N-terminal residues, we examined whether or not the binding of sarcotoxin IA to LPS abolishes the biological activities of the latter. It is known that LPS activates mouse macrophage-like cell line J774.1 to produce TNF-α (*13*). For this, LPS has to bind to J774.1 cells. We examined the effects of native and mutant sarcotoxin IA on the binding of LPS to J774.1 cells. As shown in Fig. 4, increasing amounts of native sarcotoxin IA, but no mutant sarcotoxin IA, progressively inhibited the binding of 1 μg of FITC-LPS to 2×10^6 J774.1 cells. These results indicate that sarcotoxin IA has affinity to LPS, and that sarcotoxin IA-bound LPS loses the ability to bind to J774.1 cells. To further confirm these results, we measured TNF-α produced by J774.1 cells in the presence of LPS. As shown in Fig. 5, native sarcotoxin IA dose-dependently inhibited the production of TNF-α by J774.1 cells in the presence of LPS, but no appreciable inhibition of TNF-α production was detected with mutant sarcotoxin IA. Similar experiments were performed with mouse peritoneal macrophages, and it was confirmed that no appreciable TNF-α, IL-1, IL-10, or GM-CSF was produced by macrophages when they had been treated with LPS in the presence of sarcotoxin IA (data not shown). Thus, it is clear that sarcotoxin IA–bound LPS loses its biological ability to stimulate mammalian macrophages.

As sarcotoxin IA was found to inactivate LPS, we examined what happens when sarcotoxin IA–bound LPS was introduced into the abdominal cavities of *Sarcophaga* larvae. We mixed 1 μg/ml of LPS with increasing amounts of native or mutant sarcotoxin IA, and then injected the mixtures into the abdominal cavities of third instar larvae. The

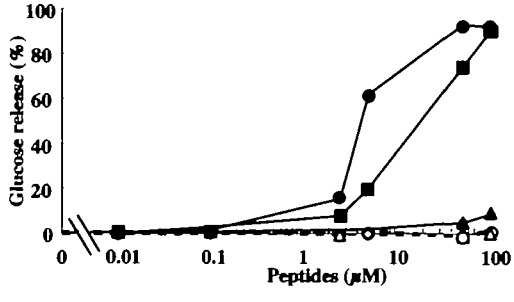

Fig. 3. **Disruption of liposomes by sarcotoxin IA.** Liposomes were prepared with a fixed amount of PC and increasing amounts of lipid A. The release of glucose entrapped in the liposomes as a percentage was plotted against the concentration of sarcotoxin IA added to the reaction mixture. The amount of glucose released on treatment of the liposomes with 0.1% (v/v) Triton X100 was taken as 100%. Closed and open symbols indicate native and mutant sarcotoxin IA, respectively. The lipid A contents of the liposomes [% (w/w)] were: ◆ and ◇, 0; ▲ and △, 1; ■ and □, 10; ● and ○, 50. Means of duplicate measurements were shown.

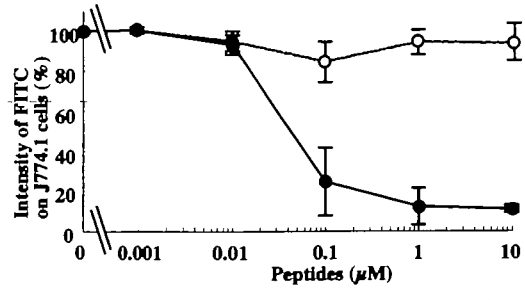

Fig. 4. **Binding of LPS to J774.1 cells.** Cells were incubated with 1 μg/ml of FITC-LPS for 1 h in the presence of increasing amounts of sarcotoxin IA, the intensity of the bound FITC as a percentage was plotted against the concentration of sarcotoxin IA added as a competitor. Binding of FITC-LPS without a competitor was taken as 100%. ●, native sarcotoxin IA; ○, mutant sarcotoxin IA. Means of quadruplicate measurements were shown with standard deviations.

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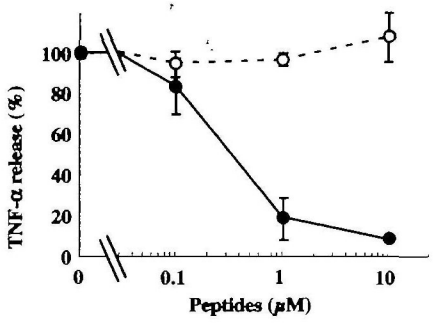

Fig. 5. **Inhibition of TNF-α production by sarcotoxin IA.** J774.1 cells were incubated with LPS (250 ng/ml) for 6 h in the presence of various concentrations of native ● or mutant sarcotoxin IA ○, and then TNF-α produced in the culture medium was measured by ELISA. Means of quadruplicate measurements were shown with standard deviations.

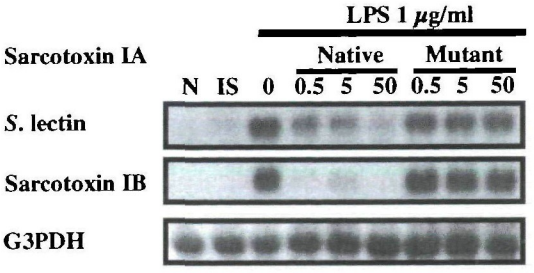

Fig. 6. **Northern blot analysis of total RNA from *Sarcophaga* larvae.** Third instate larvae of *Sarcophaga* were injected with mixtures of 1 μg/ml of LPS and increasing amounts of sarcotoxin IA. Total RNA was extracted 9 h later, and then subjected to Northern blotting hybridization using *Sarcophaga* lectin and sarcotoxin IB cDNA probes. The same filter was hybridized with G3PDH cDNA to assess the efficiency of RNA extraction. N, naive larvae; IS, insect saline-injected larvae. The figures at the top indicate the amounts of native or mutant sarcotoxin IA injected in μM.

larvae were kept for 9 h at 27°C. Then total RNA was extracted from the whole bodies and subjected to Northern blotting, using DNA fragments of the coding regions of the genes for *Sarcophaga* lectin and sarcotoxin IB as probes (*2, 12*). These are typical defense molecules of this insect. As is evident from Fig. 6, injection of LPS alone clearly induced expression of these genes. However, increasing amounts of native sarcotoxin IA, but not mutant sarcotoxin IA, progressively inhibited the expression of these genes. These results indicate that sarcotoxin IA-bound LPS is inactive, and that it cannot activate the defense system of this insect. Possibly, sarcotoxin IA firmly binds to LPS, and the resulting complex is stable even in the insect hemolymph, keeping LPS inactive *in situ*.

DISCUSSION

In this study, we demonstrated that the two N-terminal residues of sarcotoxin IA are essential for its antibacterial activity. Judging from the results for cecropin A (*7*), Trp at position 2 is crucial and the N-terminal Gly may not be directly related to its antibacterial activity. It was shown, for the first time, that these two residues are essential for sarcotoxin IA to bind to LPS (Fig. 2). Thus, the loss of antibacterial activity of mutant sarcotoxin IA can be ascribed to its loss of LPS binding activity. Binding between sarcotoxin IA and LPS is not simply explained by their ionic interaction, although sarcotoxin IA is a positively charged peptide and LPS a negatively charged molecule. Because, it is hard to expect that removal of the two N-terminal residues affects the overall charge of sarcotoxin IA (*1*). These two residues are flexible and may directly interact with lipid A to promote the formation of a tight complex of sarcotoxin IA and LPS. Incidentally, we previously found that these two residues are not included in the N-terminal α-helix of sarcotoxin IA (*14*).

Only native sarcotoxin IA made entrapped glucose permeable to PC liposomes when lipid A was integrated into liposomes. Previously, we demonstrated that sarcotoxin IA interacted with liposomes containing acidic phospholipids, releasing entrapped glucose (*10*). Taking these facts together, we speculate that the interaction between acidic phospholipids and sarcotoxin IA is rather non-specific, and simply depends upon their electrochemical charges. In fact, we found that both native and mutant sarcotoxin IA interacted with liposomes having phospholipid compositions of PG:CL = 7.5:2.5 and PE:PG:CL = 7:2:1, and released entrapped glucose in the same way (PG, phosphatidylglycerol; CL, cardiolipin; PE, phosphatidylethanolamine; data not shown). On the other hand, the interaction between lipid A and sarcotoxin IA is more specific and requires the two N-terminal residues. There seems to be no ionic interaction between mutant sarcotoxin IA and lipid A, although the former is a cationic peptide and the latter an anionic substance.

It is known that Gram-negative bacteria are more sensitive to sarcotoxin IA than Gram-positive bacteria (*5*). Possibly, sarcotoxin IA binds to both LPS and acidic phospholipids of bacterial membranes, causing membrane perturbation. However, damage caused by non-specific binding of sarcotoxin IA to acidic phospholipids may be less effective than that caused by specific binding of sarcotoxin IA to LPS.

It was found that sarcotoxin IA abolished the biological activities of LPS. Namely, sarcotoxin IA–bound LPS was found to lose the ability to bind to the surface of mouse macrophages, and thus the ability to activate the macrophage to produce various cytokines including TNF-α. It is proposed that LPS first binds to LPS-binding protein (LBP) in the serum and the resulting complex interacts with CD14 (*15, 16*), which may recruit Toll-like receptor 4. Although FITC-LPS alone bound to J774.1 cells, its binding was inhibited in the presence of sarcotoxin IA (Fig. 4). Clearly, this is due to preferential binding of sarcotoxin IA to LPS, since mutant sarcotoxin IA did not interfere with the binding of FITC-LPS to J774.1 cells. We are not certain to what extent serum LBP participates in the binding of LPS to J774.1 cells in our experimental system. Possibly, LPS directly interacts with CD14 and sarcotoxin IA–bound LPS may not be able to bind to CD14 regardless of serum LBP.

We have not characterized sarcotoxin IA–bound LPS, but binding between sarcotoxin IA and LPS is stable even in the hemolymph of *Sarcophaga* larvae, since sarcotoxin IA-bound LPS was found to lose the ability to activate the defense protein genes of this insect when introduced into the larval body cavity. In insects, LPS is known to activate defense protein genes (*17*), and LBP plays a crucial roles in

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this process (*18*). Probably, sarcotoxin IA-bound LPS is not able to bind to LBP.

It is known that bacterial LPS causes endotoxin shock (*19*). To treat endotoxemia, substances that neutralize LPS activity are needed. Polymixin B, a small cyclic peptide, binds to and neutralizes LPS, but its clinical use is precluded because of its toxicity (*20*). Recently, several cationic peptides, including cecropin family peptides, have been proposed to neutralize LPS activity (*21–25*). Our findings indicate that the LPS-neutralizing activity of sarcotoxin IA is not simply due to its cationic charge, but to its two N-terminal residues. It may be possible to design potent anti-LPS peptides based on sarcotoxin IA and related peptides. Released LPS may be also toxic to insects. It is remarkable that sarcotoxin IA plays dual roles in killing Gram-negative bacteria and detoxifying released LPS in the hemolymph of *Sarcophaga*.

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