Eosinophil Cationic Protein High-Affinity Binding to Bacteria-Wall Lipopolysaccharides and Peptidoglycans†

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Received October 15, 2007; Revised Manuscript Received December 24, 2007

ABSTRACT: The eosinophil cationic protein (ECP) is an eosinophil-secreted RNase involved in the immune host defense, with a cytotoxic activity against a wide range of pathogens. The protein displays antimicrobial activity against both Gram-negative and Gram-positive strains. The protein can destabilize lipid bilayers, although the action at the membrane level can only partially account for its bactericidal activity. We have now shown that ECP can bind with high affinity to the bacteria-wall components. We have analyzed its specific association to lipopolysaccharides (LPSs), its lipid A component, and peptidoglycans (PGNs). ECP high-affinity binding capacity to LPSs and lipid A has been analyzed by a fluorescent displacement assay, and the corresponding dissociation constants were calculated using the protein labeled with a fluorophor. The protein also binds *in vivo* to bacteria cells. Ultrastructural analysis of cell bacteria wall and morphology have been visualized by scanning and transmission electron microscopy in both *Escherichia coli* and *Staphylococcus aureus* strains. The protein damages the bacteria surface and induces the cell population aggregation on *E. coli* cultures. Although both bacteria strain cells retain their shape and no cell lysis is patent, the protein can induce in *E. coli* the outer membrane detachment. ECP also activates the cytoplasmic membrane depolarization in both strains. Moreover, the depolarization activity on *E. coli* does not require any pretreatment to overcome the outer membrane barrier. The protein binding to the bacteria-wall surface would represent a first encounter step key in its antimicrobial mechanism of action.

The eosinophil cationic protein (ECP)[1] is an antimicrobial RNase that participates in the immune host defense (*1*). The protein is involved in inflammatory processes mediated by eosinophils (*2, 3*), and its levels in biological fluids are used as a clinical marker for the monitoring of inflammatory diseases (*4*). ECP displays some immunomodulation properties (*2*) and is also toxic on the host epithelial tissues. In fact, ECP is cytotoxic for tracheal epithelium (*5*), and ECP deposits, related to tissue damage, are observed after eosinophil degranulation in inflammatory disorders (*6*). A local toxic action, following eosinophil degranulation at the epithelium level, could depend upon the protein direct interaction with the cell membranes and the extracellular matrix (*7, 8*). ECP possesses bactericidal, antiviral, and antiparasitic activities and inhibits mammalian cell growth. Its ribonucleolytic activity with common RNA substrates is low (*3*) and does not appear to be necessary for the antibacterial capacity (*9*). ECP bactericidal activity is not shared by the other eosinophil ribonuclease close homologue EDN nor by their common EDN/ECP "ancestral" precursor (*10*). The mammalian RNases family study indicates that EDN and ECP emerged via a gene duplication and would have respond to unusual evolutionary constraints, which would have promoted an increased cationicity and cytotoxicity for ECP (*10–12*). ECP sequence has indeed 19 Arg residues, providing the mature protein a pI higher than 11.

ECP bactericidal activity can be related to its membrane disruption capacity. ECP can permeabilize the outer and inner *Escherichia coli* membranes (*13*) and was reported to create transmembrane porus in synthetic lipid vesicles (*14*). In fact, ECP bactericidal activity correlates with its membrane disruption capacity and is dependent upon both surface exposed hydrophobic and cationic residues (*15, 16*). ECP can bind and partially insert into the lipid bilayers, promoting the lipid vesicles aggregation and lysis, following a "carpet-like" mechanism (*17*). However, the membrane-destabilizing activity cannot solely explain the ECP antimicrobial action (*15, 16*).

The fact that the protein activity on lipid bilayers cannot fully explain its bactericidal activity (*15*) suggests that other additional key events at the bacteria cell wall are also taking place. We have now proven that ECP can specifically bind with high affinity to lipopolysaccharides (LPSs) and peptidoglycans (PGNs). We have also analyzed the protein action

† This project was funded by a grant from the Ministerio de Educación y Cultura (Grants BMC2003-08485-C02-01 and BFU2006-15543-C02-01) and the "Fundació La Marató de TV3" (TV3-031110). M.T. is the recipient of a predoctoral fellowship from the Generalitat de Catalunya.

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[1] Abbreviations: ECP, eosinophil cationic protein; BC (BODIPY TR cadaverine), (5-(((4-(4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-yl)phenoxy)acetyl)amino)pentylamine, hydrochloride; BSA, bovine serum albumin; $DiSC_3(5)$, 3,3-dipropylthiacarbocyanine; ED_{50}, effective displacement value; LPS, lipopolysaccharide; PGN, peptidoglycan; PMB, polymyxin B; PGRP, peptidoglycan recognition protein; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

10.1021/bi702065b CCC: $40.75
Published on Web 02/23/2008

on the bacteria population by electron microscopy and its cytoplasmic membrane depolarization capacity. The characterization of the ECP-binding capacity to bacteria-wall components can contribute to the identification of the host pattern recognition domains for bacteria wall. Although there is a rich bibliography on the antimicrobial peptides action on lipid bilayers, less is known on the first recognition step at the bacteria surface. Some antimicrobial peptides have been reported to bind to bacteria-wall components, and the action at the cell surface layer is, for many antibiotics, a key event on the bacteria-killing process (*18–20*). The screening for specific interaction domains to bacteria unique features is of great interest for the drug-design industry for the development of directed immunotherapies and the treatment of autoimmune diseases (*21*).

EXPERIMENTAL PROCEDURES

Materials. BODIPY TR cadaverine, BC [5-(((4-(4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-yl)phenoxy)acetyl)amino)pentylamine, hydrochloride] and 3,3-dipropylthiacarbocyanine [diSC$_3$(5)] were purchased from Molecular Probes (Eugene, OR). LPSs from *E. coli*serotype 0111:B4, lipid A diphosphoryl from *E. coli* serotype F583 (Rd mutant), and lysozyme from chicken egg white were purchased from Sigma-Aldrich (St. Louis, MO). Polymyxin B (PMB) sulfate, peptidoglycans from *Staphylococcus aureus*, and *Micrococcus lysodeiticus* lyophilized cells were purchased from Fluka (Sigma-Aldrich). Gramicidin D and Alexa Fluor 488 protein labeling kit were purchased from Molecular Probes (Eugene, OR). *E. coli* BL21DE3 (Novagen, Madison, WI) and *S. aureus* 502 A (ATCC, Rockville, MD) strains were used. PD-10 columns were purchased from GE Healthcare.

Expression and Purification of Recombinant ECP. Wild-type ECP was expressed using a synthetic gene for human ECP coding sequence (*22*). Protein expression in *E. coli* BL21(DE3) strain (Novagen, Madison, WI), folding of the protein from inclusion bodies, and the purification steps were carried out as previously described (*22*).

Fluorescent Labeling of ECP. ECP was labeled with the Alexa Fluor 488 fluorophor, following the instructions of the manufacturer. To 0.5 mL of a 2 mg/mL protein solution in phosphate saline buffer (PBS), 50 μL of 1 M sodium bicarbonate at pH 8.3 was added. The protein is incubated for 1 h at room temperature, with the reactive dye, with stirring, following the conditions of the manufacturer. The labeled protein was separated from the free dye by a PD10-desalting column.

Fluorescent Probe Displacement Assay for LPS and Lipid A Binding to ECP. LPS binding was assessed using the fluorescent probe BODIPY TR cadaverine (BC) as previously described (*23*). BC binds strongly to native LPSs, specifically recognizing the lipid A portion. When a protein that interacts with LPSs is added, BC is displaced from the complex and its fluorescence is increased, decreasing its occupancy factor. LPS-binding assays were carried out in a 5 mM Hepes buffer at pH 7.5. The displacement assay was performed by the addition of 1–2 μL aliquots of a solution of PMB, ECP, or RNase A to a 1 mL of a continuously stirred mixture of either LPSs (10 μg/mL) or lipid A (10 μg/mL) and BC (10 μM) in a quartz cuvette. Fluorescence measurements were performed on a Cary Eclipse spectrofluorimeter. The BC excitation wavelength was 580 nm, and emission wavelength was 620 nm. Excitation slit was set at 2.5 nm, and emission slit was set at 20 nm. Final values correspond to an average of four replicates and were the mean average value of a 0.3 s continuous measurement. Quantitative effective displacement values (ED_{50}) were calculated. The ED_{50} was computed at the midpoint of the fluorescent signal versus the protein concentration of the displacement curve by a curve-fitting of the data to the equation

$$\mathrm{OF} = \frac{F_0 - F}{F_0 - F_{\max}}$$

where OF is the occupancy factor, F_0 the fluorescence intensity of BC alone, $F_{\max}$ is the intensity in the presence of lipid A or LPSs at a saturation concentration, and F is the intensities of the lipid A/BC or LPS/BC mixtures at each displacer concentrations. PMB and RNase A were used as positive and negative controls, respectively.

Affinity Binding Assay for LPSs, Lipid A, and PGN Binding to ECP. Alternatively, a titration assay was performed using the ECP labeled with the Alexa Fluor 488 fluorophor, and the K_d for the protein binding to both LPSs and lipid A was calculated. Aliquots of 100 μL of either LPSs or lipid A at 5 μg/mL in PBS were coated on flexible 96-well microtiter plates overnight at 4 °C with constant shaking. The liquid was removed, and the wells were washed extensively with PBS, blocked by incubation with 1% bovine serum albumin (BSA) for 1 h at 37 °C, washed once more with PBS, and incubated with the Alexa Fluor 488-labeled ECP in PBS overnight at 4 °C with constant shaking. Plates were read with a fluorescence multiplate reader (Victor 3, Perkin-Elmer) using the excitation filter P490 and the emission filter F535.

ECP binding to PGNs was analyzed by a fluorescence-based method, using a microtiter plate. ECP labeled with the fluorophor Alexa Fluor 488 was incubated with insoluble peptidoglycans. ECP at different concentrations, from 1 to 100 nM, was incubated in the presence of 0.02 μg of peptidoglycans in a 5 mM Hepes buffer at pH 7.5 in a final volume of 200 μL. The reaction mixture was kept with shacking for 2 h at 4 °C. Afterward, the remaining soluble protein was removed from the PGN-insoluble fraction by a centrifugation step at 13000*g* for 30 min and quantified with a Victor 3 reader (Perkin-Elmer).

ECP Binding to PGNs by Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). Peptidoglycans at 0.4 mg/mL in 10 mM Tris-HCl at pH 7.5 were incubated with ECP at 0.02 mg/mL in a protein/PGN relation of 1:20 (w/w). Samples were kept for 2 h at 4 °C with gentle mixing and centrifuged at 13000*g* for 15 min to separate the soluble and insoluble fractions. Lysozyme and BSA were chosen as positive and negative controls, respectively. Samples were resuspended directly in the electrophoresis loading buffer, and the presence of the protein in the soluble and insoluble fractions was evaluated using an Experion automated microfluidic electrophoresis system (BioRad).

Protein Binding to Bacteria Cells. ECP was incubated at 4 μM with bacteria cells (*E. coli* and *S. aureus*) grown to the exponential fase (OD_{600} = 0.6) in 0.1 mL of 10 mM phosphate buffer at pH 7.5 and 37 °C for 30 min. After centrifugation at 13000*g*, aliquots of the pellet and liophyl-

ized supernatant fractions were resuspended in the electrophoresis loading buffer and analyzed by SDS−PAGE and Coomassie Blue staining.

Scanning Electron Microscopy (SEM). *E. coli* and *S. aureus* cell cultures of 1 mL were grown at 37 °C to mid-exponential phase (OD_{600} of 0.4) and incubated with 4 μM ECP in phosphate-buffered saline (PBS) at room temperature. Sample aliquots were taken up to 4 h of incubation and were prepared for analysis by SEM, as previously described (*24*). The cell suspensions were fixed with 2.5% gluteraldehyde in 100 mM Na-cacodylate buffer at pH 7.4 for 2 h at room temperature. Afterward, the cells were pelleted, a drop of each suspension was transferred to a nucleophore filter, which was kept for 30 min in a hydrated chamber for the cells to adhere, then washed to remove the gluteraldehyde, and resuspended in the same buffer 100 mM Na-cacodylate buffer at pH 7.4. Attached cells were post-fixed by immersing the filters in 1% osmium tetroxide (OsO_4) in cacodylate buffer for 30 min, rinsed in the same buffer, and dehydrated in ethanol in ascending percentage concentrations [30, 70, 90 (2×), and 100 (2×)], for 15 min each. The filters were mounted on aluminum stubs and coated with gold−palladium in a sputter coater (Emitech K550). The filters were viewed at 15 kV accelerating voltage in a Hitachi S-570 field emission scanning electron microscope, and a secondary electron image of cells for topography contrast was collected at several magnifications. A total of 10 micrographies were collected at random for each condition, and the number of isolated cells and aggregates were registered.

Transmission Electron Microscopy (TEM). Mid-logarithmic-phase *E. coli* and *S. aureus* cells ($OD_{600} \sim 0.4$) were incubated with 4 μM ECP for 3 h. After treatment, bacterial pellets were prefixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer at pH 7.4 for 2 h at 4 °C and post-fixed in 1% osmium tetroxide buffered in 0.1 M cacodylate at pH 7.4 for 2 h at 4 °C. The samples were dehydrated in acetone (50, 70, 90, 95, and 100%). The cells were immersed in EPON resin, and ultrathin sections were examined in a JEOL JEM 2011 (Jeol Ltd., Tokio, Japan).

Detection of PGN-Soluble Products. The potential release of soluble PGN fragments, as digestion products from *S. aureus* murein layer, was assessed by a colorimetric assay. Soluble *N*-acetylhexosamines were quantified by a modified Morgan−Elson reaction, as previously described (*25*). The digestion of *M. lysodeiticus*-lyophilized cells by lysozyme was taken as a positive reference control.

S. aureus cell cultures of 1 mL were grown at 37 °C to mid-exponential phase (OD_{600} of 0.4) and incubated overnight with 4 μM ECP in PBS at room temperature. Insoluble material was removed by centrifugation for 30 min at 13000*g*, and soluble material was transferred to another Eppendorf tube. A total of 100 μL of the soluble digestion products were mixed with 100 μL of 2% $K_2B_4O_7$ in water. The samples were boiled for 30 min and allowed to cool to room temperature. *P*-Dimethylaminobenzaldehyde (Ehrlich's reagent) was dissolved into a total volume of 9.5 mL of acetic acid, and 0.5 mL of concentrated HCl was added to give a stock reagent. A total of 1 mL of stock reagent was diluted per 7 mL of acetic acid to generate the color reagent. To the boiled sample, 900 μL of the color reagent was added, and the mixture was kept at 37 °C for 20 min. Color was read spectrophotometrically at 585 nm, and samples were compared to standards of *N*-acetylglucosamine of known concentration. *N*-Acetylglucosamine standards were boiled in $K_2B_4O_7$ for 7 min instead of 30 min to compensate known discrepancies between the extinction coefficient of free *N*-acetylhexosamines and those that exist as disaccharides or are amidically linked to wall peptides.

Bacteria Cytoplasmic Membrane Depolarization Assay. Membrane depolarization was followed using a modification of a previous reported assay (*26*), using the $DiSC_3(5)$ lipophilic dye that changes its fluorescence intensity in response to changes in transmembrane potential. *E. coli* and *S. aureus* cells were grown at 37 °C to mid-exponential phase ($OD_{600} = 0.4$), centrifuged at 5000*g* for 7 min, washed with 5 mM Hepes at pH 7.2 containing 20 mM glucose, and resuspended in 5 mM Hepes-KOH, 20 mM glucose, and 100 mM KCl at pH 7.2 to an OD_{600} of 0.05. $DiSC_3(5)$ was added to a final concentration of 0.4 μM. Changes in the fluorescence because of the alteration of the cytoplasmic membrane potential were continuously monitored at 20 °C using a spectrofluorimeter at an excitation wavelength of 620 nm and an emission wavelength of 670 nm. When the dye uptake was maximal, as indicated by a stable reduction in the fluorescence because of quenching of the accumulated dye in the membrane interior, protein in 5 mM Hepes-KOH buffer at pH 7.2 was added at a final tested protein concentration of 4 μM. Gramicidin D was used as control reference protein. All conditions were assayed in duplicate. The time necessary to reach a stabilized maximum fluorescence reading was recorded for each condition, and the time required to achieve half of total membrane depolarization was estimated from the nonlinear regression curve.

E. coli cells were also incubated in the presence of ethylenediaminetetraacetic acid (EDTA), to lose the LPS outer membrane surface layer, as previously described (*27*). Gram-negative bacteria cells treated with EDTA were used to better characterize the protein membrane depolarization capacity in the absence of a structured LPS layer. Briefly, cells were grown at 37 °C to mid-exponential phase, centrifuged at about 5000*g* for 7 min, washed with 5 mM Hepes at pH 7.2 containing 20 mM glucose 2 mM EDTA, washed with 5 mM Hepes at pH 7.2 containing 20 mM glucose, and resuspended in Hepes buffer (5 mM Hepes at pH 7.2, 20 mM glucose, and 100 mM KCl) to an OD_{600} of 0.05.

RESULTS

ECP Binding to LPSs and Lipid A. The use of a new optimized fluorescent method for a high- throughput screening of LPS-binding molecules (*23*) has enabled us to easily assess the ECP−LPS affinity interaction. The method is highly sensitive and uses, as a fluorescent probe, a derivative of cadaverine [BODIPY TR cadaverine (BC)], which specifically binds to the lipid A portion of the LPSs (*28*). BC is a dansyl cadaverine derivative, which can be used as a sensitive and robust fluorescent probe optimized for the quantification of the LPS and lipid A specific interaction. The probe would bind mainly through salt- bridge interactions between the free protonable group of cadaverine and the phosphates of the lipid A glycosidic portion (*23, 28*). The assay monitors

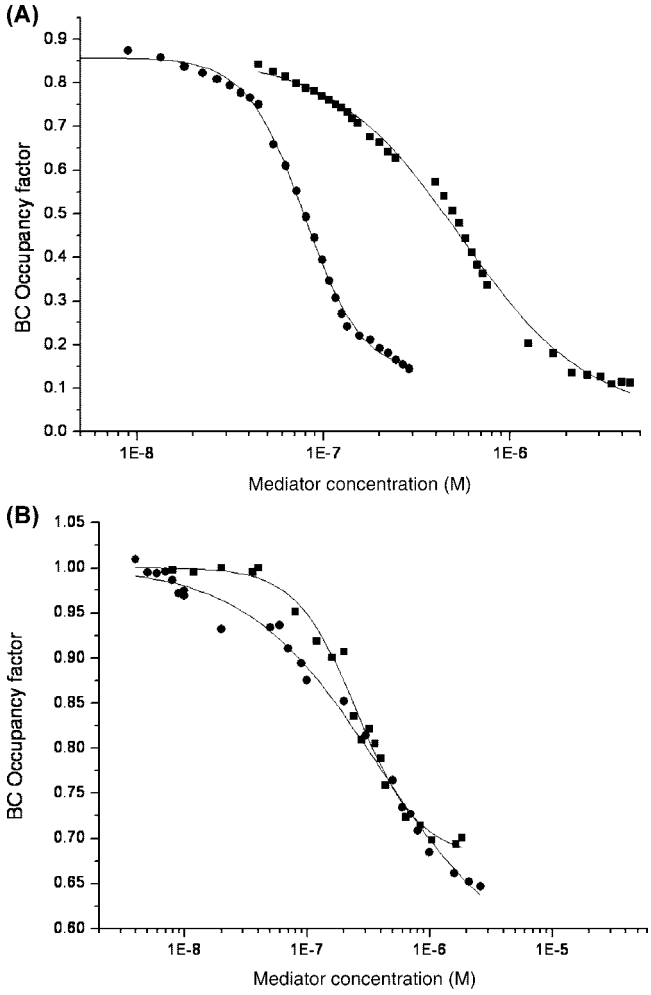

FIGURE 1: (A) Displacement of LPS-bound BC by ECP (●) and polymyxin B (■). [LPS], 10 μg/mL; [BC], 10 μM in 5 mM Hepes-KOH at pH 7.5. (B) Displacement of lipid A-bound BC by ECP (●) and polymyxin B (■). [Lipid A], 20 μg/mL; [BC], 10 μM in 5 mM Hepes-KOH at pH 7.5. The BC displacement, manifested as dequenching of the fluorescence signal, is quantified by its occupancy factor, as detailed in the Experimental Procedures.

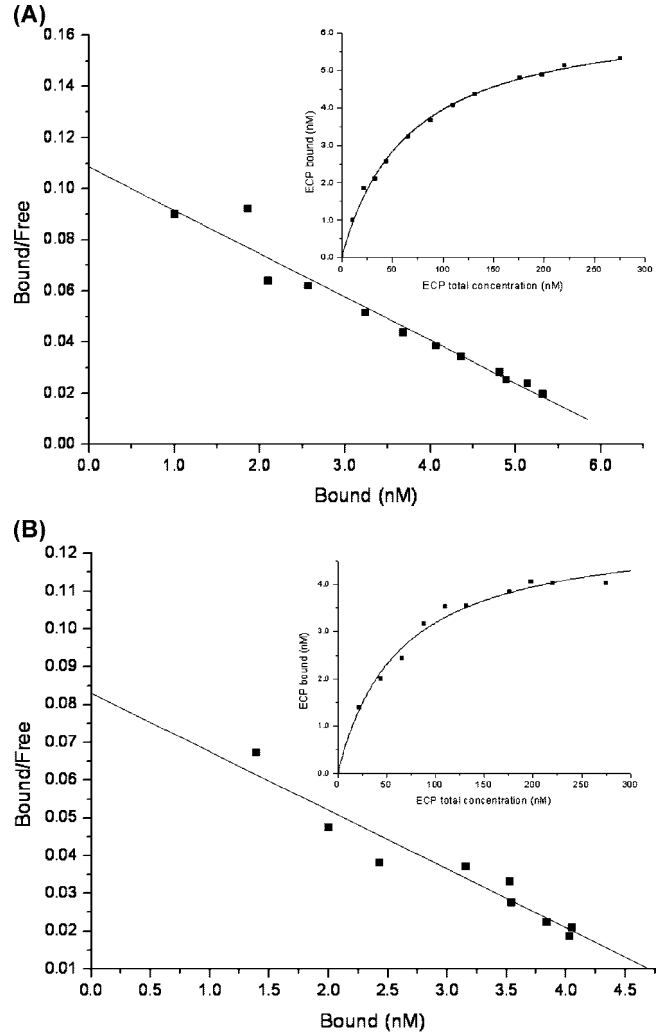

FIGURE 2: Scatchard plots and the corresponding binding curves of ECP interaction with (A) LPSs and (B) lipid A. ECP was labeled with the fluorophor Alexa Fluor 488. Aliquots of 100 μL of either LPSs or lipid A at 5 μg/mL in PBS were coated on microtiter plates, and after incubation in the protein presence, the fraction of bound ECP was quantified.

the competitive displacement of BC by other putative LPS-binding molecules.

We have used here the BC-specific assay to analyze the binding of ECP to lipopolysaccharides and the lipid A portion. The addition of ECP displaces the BC probe, and its fluorescence is increased, decreasing its occupancy factor (Figure 1). PMB, a prototype lipid A binder (*29*), was used as a positive reference control, and RNase A, an homologous mammalian RNase devoided of antimicrobial activity (*30*), was used as a negative control. Displacement of the BC-binding curves indicates that ECP is able to bind with affinity to LPSs with an even higher affinity than PMB. PMB is frequently used as a reference positive control compound for LPS-binding assays (*31*). In the other hand, the RNase A control did not show any specific binding to LPSs, as illustrated by only a reduced linear displacement (data not shown) comparable to the observed linear reduction of the cadaverine probe occupancy when increasing the solvent ionic strength by the addition of NaCl (*23*).

We have calculated the following quantitative effective displacement values for ECP binding: ED_{50} of 0.8 $\pm$ 0.1 and 3.9 $\pm$ 0.1 μM when using LPSs and lipid A, respectively. PMB ED_{50} values for LPSs and lipid A are 2.5 $\pm$ 0.1 and 2.55 $\pm$ 0.1 μM, respectively.

We observe that the calculated ED_{50} for ECP when using lipid A is similar to the values calculated for PMB. However, while polymyxin displacement capacity is comparable for either LPSs or the lipid A portion, ECP displays a significantly higher activity with LPSs than when using lipid A. In fact, the displacement capacity of ECP in the presence of LPSs is even higher than PMB.

Additionally, a titration assay was performed to calculate the K_d for ECP binding to LPSs and lipid A (Figure 2). Binding dissociation constants (K_d) were calculated using the Scatchard plot, obtaining a value of about 5×10^{-8} and 6×10^{-8} M for LPSs and lipid A, respectively.

Therefore, the results proved that the protein has a high-affinity binding constant to both LPSs and lipid A.

ECP Binding to PGNs. ECP high-affinity binding to PGNs was assessed by both SDS−PAGE and a titration assay. Analysis by SDS−PAGE of ECP incubated with peptidoglycans indicates that most of the ECP sample is recovered together with the PGN-insoluble fraction, as also observed for lysozyme, the positive control (Figure 3). On the contrary,

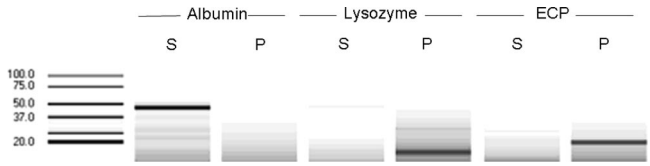

FIGURE 3: Analysis by SDS−PAGE of the binding of ECP to PGNs. Lysozyme and BSA were taken as positive and negative controls for PGN binding, respectively. For each protein, the supernatant (lane S) and pellet (lane P) fractions are included. PGNs were incubated with each protein, and the soluble and insoluble fractions were collected as described in the Experimental Procedures. Supernatant represents the soluble fraction, which contains the unbound protein, while the pellet is the insoluble fraction containing the PGN-bound protein.

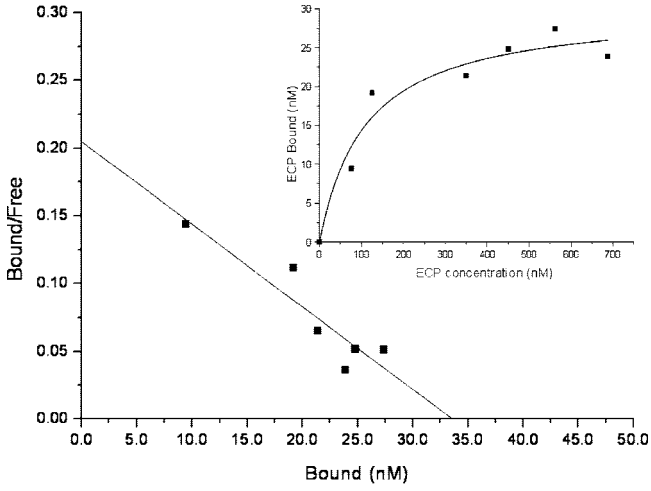

FIGURE 4: Scatchard plots and the corresponding binding curves of the ECP interaction with PGNs. ECP labeled with the fluorophor Alexa Fluor 488 at a concentration range from 0.01 to 10 nM was incubated in the presence of 0.02 μg of PGNs in 200 μL of 5 mM Hepes-KOH at pH 7.5, and the free unbound fraction was quantified.

BSA, the negative control, does not bind to the PGN fraction and is fully recovered in the supernatant fraction.

PGN-binding assay using the Alexa fluorophor-labeled ECP (Figure 4) indicates a high-affinity binding. A K_d value of 2×10^{-7} M was determined from the Scatchard plot.

ECP Binding to Bacteria Cells. ECP binds *in vivo* to the bacteria surface. After incubation of bacteria cell cultures of *E. coli* and *S. aureus* in the presence of 4 μM ECP, the pellet and supernatant fractions were analyzed by SDS−PAGE. We observe that all of the protein is recovered within the bacteria cell fraction (data not shown). A correlation between the RNase binding to bacteria cells and their bactericidal capacity is also reported for other RNases, as RNase 7 and frog RNases (*32*). ECP binding to the bacteria wall would be the first step, leading to significant changes on the *E. coli* cell surface, as visualized by electron microscopy. On the other hand, no significant changes were observed in the *S. aureus* cell wall. Likewise, no release of soluble PGN degradation products, which could have arisen from an autolysin activation mechanism, was detectable upon ECP incubation (data not shown).

ECP Activity on the Gram-Negative and Gram-Positive Bacteria Cell Population Visualized by TEM and SEM. SEM was used to follow the changes on the bacteria cell cultures upon incubation with ECP. The cell population behavior, the bacteria surface, and the cell morphology were visualized upon incubation with the protein at different time intervals.

The addition of ECP at 4 μM final concentration induces the *E. coli* cell culture aggregation. Aggregation is already observed after 45 min of protein incubation (Figure 5). Aliquots were taken up to 4 h of incubation, and no further significant differences were visualized during the studied time interval. Moreover, aggregated cells show a damaged cell envelope but keep their overall shape. The characteristic baton shape for *E. coli* cells can still be easily recognized in the cell culture aggregates, suggesting that no cell lysis is taking place.

S. aureus cells incubated with ECP in the same conditions do not show an apparent lysis process and also retain their cocci characteristic round shape (Figure 6).

From TEM, we also corroborated that most of cells in both strains retain their morphology. Details at the bacteria wall could be further identified by TEM. Separation of *E. coli* outer membrane, mostly located at the cell ends, is present in many ECP-treated cells (see arrows in parts C and D of Figure 7). Besides, TEM images also confirmed that there is no present damage at the Gram-positive cell wall, while *E. coli* cells are considerably damaged. In *E. coli* cells, we can also identify some cells that show a partial release of the internal content (see arrow in Figure 7D).

The changes visualized by electron microscopy in the bacteria cell cultures upon incubation with ECP were all performed at a protein concentration (4 μM) at which we have previously determined that the protein has a bactericidal activity (*15*).

ECP Induces the Bacteria Membrane Depolarization. The ability of antimicrobial proteins and peptides to depolarize the bacteria cytoplasmic membrane provides a direct assessment of their effect at the membrane inner level. We have proven the protein capacity to induce the bacteria membrane depolarization using a membrane potential sensitive dye. The use of the membrane potential-sensitive probe $DiSC_3(5)$ allows a continuous monitoring of the cytoplasmic membrane depolarization (*33*). The increase in the cyanine dye probe fluorescence is reported to be inversely proportional to the residual membrane potential (*34*). $DiSC_3(5)$ is added to exponential-phase cells. The dye is taken up by cells upon hyperpolarization and released upon depolarization (*33*). When the cell is hyperpolarized, the dye is recruited to the inner membrane surface, where some dye aggregation would take place and the fluorescence would be quenched. When the membrane is depolarized, a fluorescent increase is observed, as the dye is released to the medium (*33*) (Figure 8).

The ECP depolarization capacity was tested for both Gram-negative- and Gram-positive-studied strains. A significant increase in the fluorescent signal is observed right after the protein addition, indicating that ECP is active on both cases. Comparative results indicate that the depolarization capacity is higher in the Gram-negative strain tested, probably because of the easier access of the protein to the cytoplasmic membrane (Table 1).

Gram-positive assays are performed using the *S. aureus* 502 A strain. This is a well-characterized strain, regularly used in the study of antimicrobial peptides for both action at the cytoplasmic membrane and bacteria cell wall levels (*18*).

For Gram-negative assays, the *E. coli* BL21(DE3) strain was used. We did not use an outer-membrane-barrier-defective *E. coli* mutant (*34, 35*) as required for some

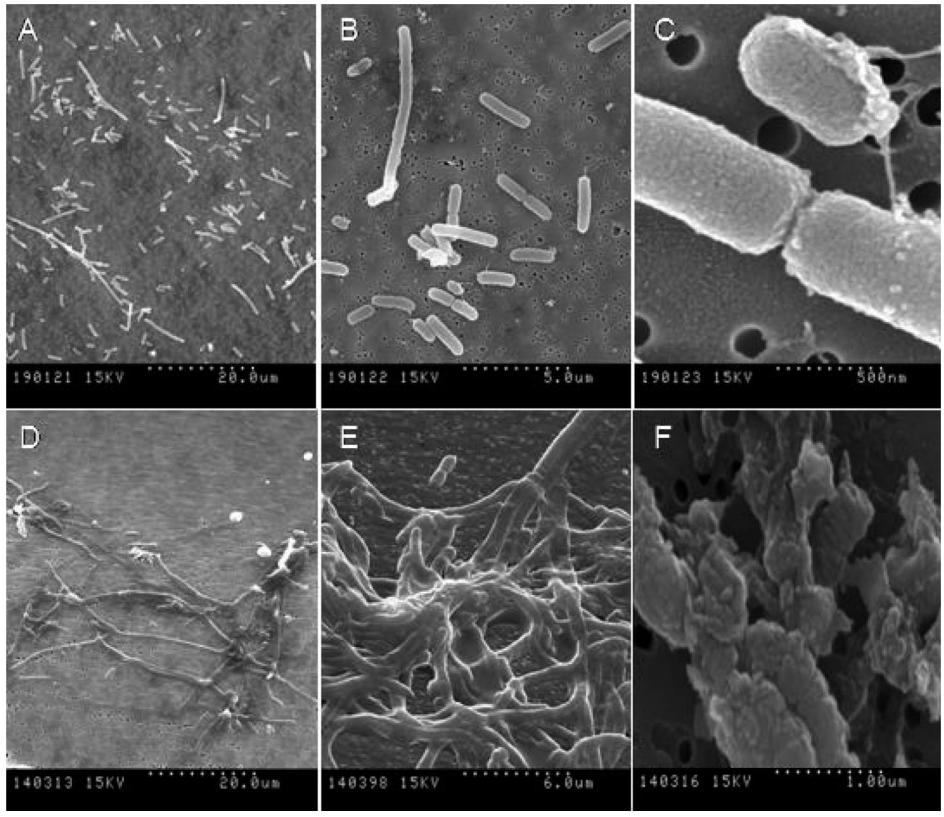

FIGURE 5: Scanning electron micrographs of *E. coli* incubated in the absence (A−C) and presence (D−F) of 4 μM ECP for 45 min. Magnification scale is indicated at the bottom of each micrograph.

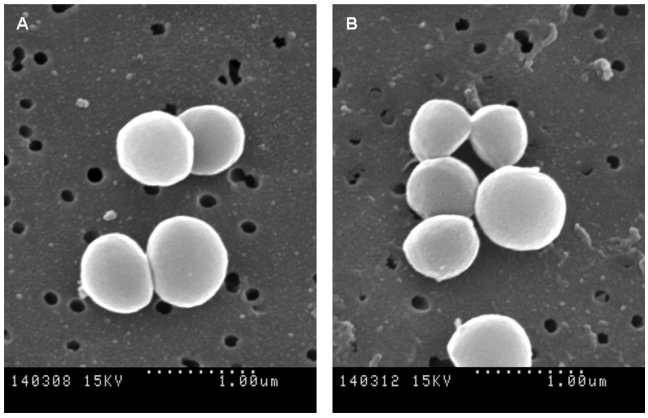

FIGURE 6: Scanning electron micrographs of *S. aureus* incubated in the absence (A) and presence (B) of 4 μM ECP for 4 h. Magnification scale is indicated at the bottom of each micrograph.

antimicrobial agents. To compare the ECP activity in the absence of the bacteria outer membrane, EDTA-treated cells were prepared. The EDTA treatment chelates the divalent cations that participate in the structured packing of the LPS layer and leads to the outer membrane disorganization. This protocol has been proven useful (*27, 36*) to assess directly the cytoplasmic membrane depolarization activity on Gram-negative strains, for antimicrobial agents with no direct action at the outer LPS layer. Our results indicate that no significant differences are detected between EDTA-treated and untreated cells upon ECP incubation (Table 1), suggesting that ECP can destabilize the LPS layer and no EDTA treatment is required to allow for the protein effect at the cytoplasmic membrane. ECP-observed behavior would be equivalent to the reported results for PMB, which is used as a positive control for the outer membrane permeabilization capacity (*37*). On the contrary, the positive control for membrane depolarization (gramicidin D) improved its depolarization activity on the EDTA-treated cells, indicating that this peptide cannot destabilize the outer membrane layer. Besides, gramicidin displays a higher depolarization activity on the tested Gram-positive strain as previously described (*18*). In fact, gramicidin is a short very hydrophobic β-sheet peptide that can form transmembrane pores and ionic channels (*38*), and its lethal mechanism is believed to involve the cytoplasmic membrane permeabilization by the formation of cation-specific channels (*39*).

Our results indicate that ECP, as a cationic polypeptide, with a high affinity for LPSs, would have an outer membrane destabilization capacity. The protein would directly destabilize the outer LPS-structured layer, facilitating its effect toward the cytoplasmic membrane. In fact, electronic micrographs (Figures 5 and 7) indicate that the *E. coli* bacteria wall is considerably damaged, and local detachment of the outer membrane is frequenly shown, after exposure to the protein.

DISCUSSION

ECP is a RNase expressed in eosinophils and stored in the secretion secondary granules. Eosinophils are important effector cells in the immune defense system. Although eosinophils are commonly associated with parasitic infections, they can additionally participate in other antimicrobial activities (*40*). When studying the ECP antibacterial activity, we must always keep in mind that thus far there is no evidence of the involvement *in vivo* of eosinophils in the host response against bacterial infections. Contribution of

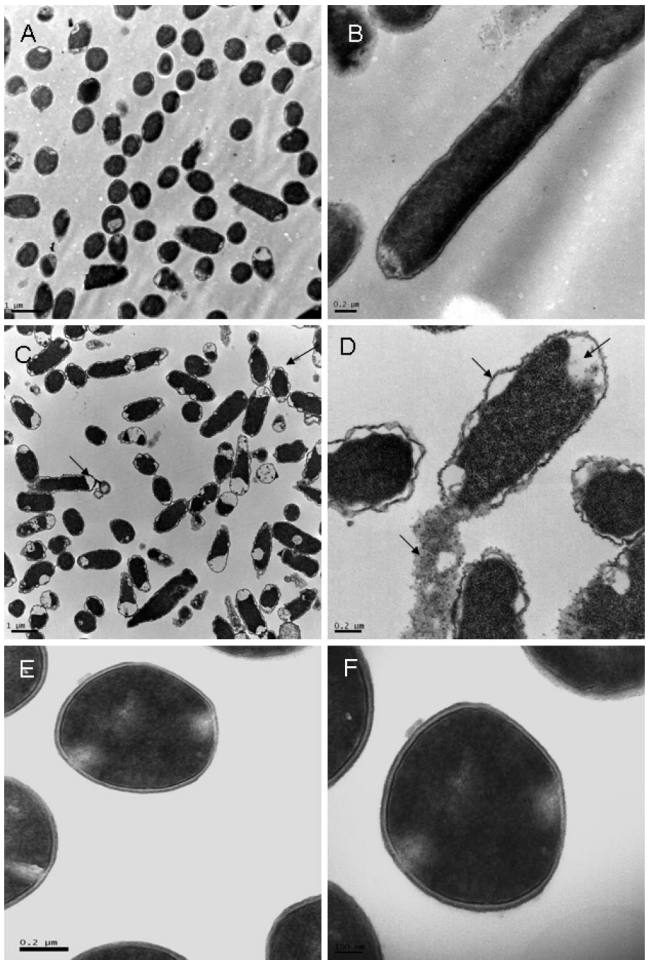

FIGURE 7: TEM of *E. coli* and *S. aureus* cells incubated with ECP. (A and B) *E. coli* control cells. (C and D) *E. coli* cells incubated with 4 μM ECP for 3 h. (E) *S. aureus* control cells. (F) *S. aureus* cells incubated with 4 μM ECP for 3 h. Magnification scale is indicated at the bottom of each micrograph.

eosinophils and their secretion proteins in the host response against bacterial infections is still a controversial issue. Some authors report antibacterial activities for eosinophils, together with neutrophils participation (*41, 42*). In fact, ECP is also detected in neutrophils (*43, 44*) and can be expressed by activated neutrophils (*45*). As neutrophils develop an active role in bacterial infections, by ingesting and killing the invading microbes (*46*), and represent a much higher percentage of circulating blood leukocytes, the active secretion of ECP by neutrophils should also make us reconsider its potential antimicrobial function. Moreover, even if the eosinophil count of peripheral blood is not typically raised in bacterial infection, eosinophils may be recruited to the infection focus. The activation of eosinophils at the inflammation tissues could account for the reported increased levels of eosinophil secretion proteins in the serum of patients with bacterial infection (*47*). Eosinophil activation and an increase in ECP secretion are also triggered in the presence of the LPS endotoxin (*45, 48*), indicating either an eosinophil response to bacterial infection or a contribution in the host allergic inflammation reaction to common potential antigens.

ECP displays broad bactericidal activity spectra, with a described capacity for both Gram-negative and Gram-positive bacteria (*13, 15*). We have previously studied ECP activity on lipid bilayers (*15, 17*). However, the protein membrane destabilizying activity does not solely explain its bactericidal capacity (*15*). We have now analyzed its capacity to interact with the bacteria cell wall.

Much effort has been devoted to develop antibiotics with only membrane disturbance activity, with not much success achieved until now (*49, 50*). A determinant factor, such as the interaction to the bacteria wall layer, was often disregarded (*19*). In fact, action at the cell surface increases the cytoplasmic membrane susceptibility to antimicrobial agents (*18*).

Polycations can possess a strong bactericidal (*51*), bacteriolysis, and autolysin-inducing activities (*52*). One of the determinant steps in the killing mechanism of cationic polypeptides is the first interaction process at the bacteria surface wall. In fact, many strains, such as *S. aureus*, have developed distinct strategies, such as the reduction of their overall negative surface charge, to acquire a better resistance to cationic antimicrobial peptides (*18, 20*). The Gram-positive cell wall is also a potential target for defensin action, and it is suggested that defensin accumulation at the bacteria surface could activate the bacteria autolysin activity, leading to the cell death (*19*). In Gram-negative bacteria, the peptide binding to the LPS layer and the concomitant physical destabilization of the outer membrane is by itself an antimicrobial mechanism (*19*).

ECP, with a cationic and amphiphilic nature, complies the main "rules" for antimicrobial peptide definition (*21*). It has an overall positive charge, with a pI higher than 11, and contains an important proportion of hydrophobic residues. Its overall positive charge should greatly facilitate its accumulation at the polyanionic microbial cell surfaces. The specific interaction of ECP with LPSs and PGNs should contribute to its bactericidal activity and may explain some of the described protein immunomodulatory properties (*2*).

LPSs, also named as "endotoxins", represent a bacterial signature, which trigger the host innate immunity (*53*). The identification of LPS-binding domains in antimicrobial proteins would contribute to the design of new drugs that specifically target unique bacterial motives. LPS-binding molecules have an additional pharmaceutical interest as endotoxin-neutralizing molecules. However, despite the great efforts in searching for molecules that would neutralize the LPS action and prevent the sepsis shock, no therapeutic drugs are currently available (*23*).

Currently, there is still scarce available information on the LPS-binding structural determinants. In fact, high-throughput screening searching for LPS recognition motives were quite unsuccessful (*31*). PMB was chosen as the starting LPS-binding molecule (*31*). PMB is a good reference antibiotic for action at the cell-wall level that is effective mainly on Gram-negative bacteria (*18*). However, PMB is restricted as a therapeutic agent because of its toxicity to host cells (*54*). The structural search for peptides mimicking the PMB-binding capacity to lipid A, which is the minimal structural LPS element necessary for endotoxic activity (*53, 55*), indicates that not only the cationic and amphipatic features but also the peptide conformation are determinant (*56*). LPS-binding motives are reported as basic and hydrophobic residues combination streches (*57*).

The anionic and amphiphilic nature of lipid A enables it to interact with cationic and amphipatic molecules (*29*). High-throughput screening for optimal recognition and

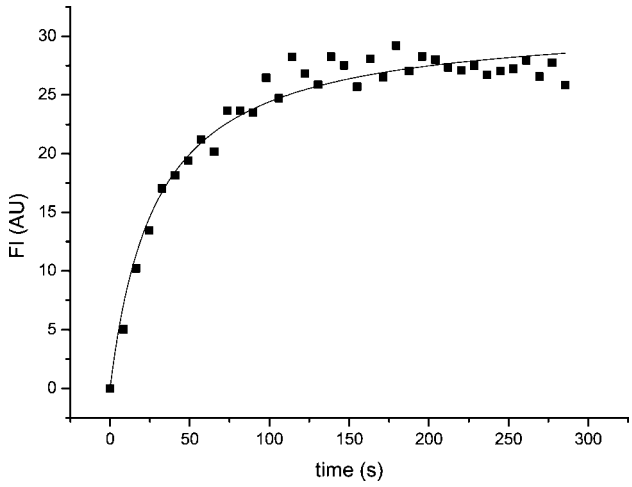

FIGURE 8: Depolarization activity of ECP on *E. coli* cells determined by the $DiSC_3(5)$ dye assay. *E. coli* cells were grown at 37 °C to mid-exponential phase ($OD_{600} = 0.4$) and resuspended in 5 mM Hepes-KOH, 20 mM glucose, and 100 mM KCl at pH 7.2 to an OD_{600} of 0.05. $DiSC_3(5)$ was added, and when the dye uptake was maximal, protein in 5 mM Hepes-KOH buffer at pH 7.2 was added at a final concentration of 4 μM. The increase in fluorescence intensity registers the dye release upon reduction of the transmembrane potential.

Table 1: Membrane Depolarization Activity

	ECP (s)	gramicidin D (s)
E. coli (−EDTA)	29 ± 2	129 ± 5
E. coli (+EDTA)	22 ± 9	75 ± 5
S. aureus	94 ± 4	39 ± 1

[a] Bacteria cytoplasmic membrane depolarization has been followed using the $DiSC_3(5)$ lipophilic dye as described in the Experimental Procedures. Membrane depolarization is followed by an increase in fluorescence intensity. Gramicidin D is used as an internal reference. Table values represent the time needed to perform half of the total membrane depolarization, as measured in seconds.

neutralization of lipid A selects molecules with two cationic groups that would interact with the negatively charged phosphates (*31*). Although the bis-cationic motif is found to be the principal determinant for the binding, an additional appropriately positioned hydrophobic group is also needed (*58*). Besides, heteroaromatic elements, as the Trp indol group, can also contribute to the binding (*59*). Some of these compounds were proven to interact and neutralize the lipid A toxicity (*31*). However, the structural data on bacteria−carbohydrate interactions with proteins is still scarce and is limited by their structural and conformational complexity (*60*). The studies are further hampered by the diverse aggregation states that LPS can adopt in different assay conditions (*58, 61*).

We have determined a very high binding affinity of ECP to LPSs. ECP could provide a useful scaffold for the analysis of the structural determinants for LPS binding. We identified previously the involvement of some cationic and hydrophobic residues in the protein activity on the lipid bilayer and bacteria viability by site-directed mutagenesis (*15, 16*). ECP arginine and hydrophobic surface residues may also contribute to the protein-binding capacity for polysaccharides.

Most of the characterized LPS-binding molecules in the literature emphasize the involvement of both cationic and hydrophobic interactions. Two well-characterized host antimicrobial proteins that belong to the family of lipopolysaccharide-binding proteins are the bactericidal permeability-increasing protein (BPI) and the lipopolysaccharide-binding protein (LBP) (*62–64*). LBP shares with BPI nearly a 50% identity at the primary structure (*64*). Conserved basic residues clustered at the N-terminal domain of both BPI and LBP are believed to mediate initial electrostatic interactions with acidic sites clustered close to the lipid A region (*64, 65*). An extended amphipatic loop is also identified for LPS binding in the horseshoe crab anti-LPS factor, which could represent a LPS-binding motif, shared together with LBP and BPI (*66*). The three-dimensional structure of the tachylectin endotoxin-binding protein reveals a hydrophobic patch and two Lys/Arg cationic clusters that could account for its lipid A-binding capacity (*66*). Lysozyme has also a high binding affinity to the lipid A portion of LPSs, in particular by the electrostatic interactions to the phosphate region (*67*). Lactoferrin also binds to LPSs (*68, 69*), and some derived peptides have been identified with LPS-neutralizing activities (*69–71*). A derived N-terminal peptide represents the minimal peptide that provides significant protection of mice against a lethal LPS challenge and demonstrates therefore its endotoxin-neutralizing capacity *in vivo* (*70*).

One of the potential mechanisms of action of cationic host defense peptides depends upon the displacement of the divalent cations that contribute to the LPS packing (*72*). The displacement would lead to a disorganization of the LPS leaflet, a concomitant disturbance at the outer membrane level, and would facilitate the entrance of the peptide (*73*). This mechanism by which antimicrobial peptides can cross the outer membrane has been named the "self-promoted uptake" pathway. The process includes as a first triggering step, the direct binding of the peptides to the LPS (*74, 75*). After the peptide association to the outer surface of the cytoplasmic membrane, we may have membrane-disruptive or nondisruptive processes (*76*). This mechanism might be important for the bactericidal activity of cationic peptides on Gram-negative strains. A direct correlation between the LPS-binding capacity, the peptide aggregation at the outer membrane, and the final killing event is reported, for example, for lactoferrin-derived peptides (*68*) and PMB (*77*). The triggering of the "self-promoted uptake" pathway by cationic polypeptides may also activate the bacteria autolysins (*52*).

ECP activity on Gram-negative strains may also depend upon the outer membrane destabilization process. In fact, electron micrographs by TEM show a damaged outer bacteria surface in ECP-treated cells (parts C and D of Figure 7). Besides, we have proven that ECP can have an effect at the Gram-negative bacteria cytoplasmic membrane, without requiring any additional treatment to remove the outer layer barrier. Our results (Table 1 and Figure 8) indicate that ECP can destabilize by itself the *E. coli* outer membrane, without an EDTA pretreatment or the use of an outer-membrane-defective mutant bacteria strain.

ECP-binding studies also indicated a high affinity toward PGNs (Figures 3 and 4). PGN are the major constituent of Gram-positive bacteria cell walls and can induce several inflammatory responses upon bacteria infection (*78*). PGNs are targeted by specific pattern recognition proteins, classified as peptidoglycan recognition proteins (PGRPs). The PGRPs are highly conserved from insects to mammals and are important contributors to the host defense system against invading bacteria (*79*). In some inflammation diseases,

the inability of the host response system to control bacteria infection may initiate an exhacerbate systemic response (*80*). Identification of PGN-binding domains is key for the understanding of some autoimmune disease mechanisms.

The binding to PGNs at the bacteria surface can trigger the cell autolysins. We have discarded any activation of the murein layer degradation by ECP action, by checking the absence of the release of PGN-soluble digestion products upon the bacteria cell culture incubation with the protein. Moreover, electron micrographs of *S. aureus* cells do not show any damage on the wall surface, and no cell lysis is detected.

To evaluate both the ECP capacity to interact at the bacteria surface and the cytoplasmic membrane levels and its capacity to exert a bactericidal action, we have further analyzed the protein action by additional methodologies. We have previously characterized in detail ECP bactericidal activity and its action on synthetic lipid vesicles as a model for the membrane lipid bilayer (*15–17*). We have confirmed here the protein capacity to induce the bacteria cytoplasmic membrane depolarization on both Gram-negative and Gram-positive strains (Table 1). Additionally, changes on the bacteria cell population behavior and the cell morphology were further analyzed by electron microscopy (Figures 5−7).

The cell culture visualization by SEM indicates that both Gram-positive and Gram-negative strains do retain their characteristic shape, even after 4 h of incubation, suggesting that no bacteria cell lysis is taking place in the assayed conditions. Nonetheless, *E. coli* ECP-treated cells aggregate and present a clear damage to the surface wall, even before 1 h of protein incubation. All of the described effects at the surface and cytoplasmic membrane levels were obtained at an ECP concentration where a bactericidal activity was previously observed for both strains (*15, 16*). Moreover, analysis at this protein concentration on lipid vesicles indicated a partial immersion of the protein on the lipid bilayer and a membrane destabilization capacity (*17*). The results suggest that the protein can exert partially or totally its antimicrobial action without requiring its internalization into the cytosol.

Our results indicate that ECP can destabilize by itself the LPS layer, having a direct access to the cytoplasmic membrane. A correlation between the outer membrane permeabilization capacity and the cytoplasmic membrane depolarization is reported for many cationic peptides (*34, 81*). Some antimicrobial agents can exert a direct effect on the cytoplasmic membrane thanks to its capacity to destabilize or break the bacteria wall. We can distinguish between antimicrobial agents, such as PMB, that can directly destabilize the outer membrane (*18, 37, 81*) and the ones that cannot destabilize the Gram-negative outer membrane and require a bacteria cell pretreatment to depolarize the cytoplasmic membrane. ECP, similar to PMB, does not require any pretreatment to show a depolarization activity. ECP capacity to induce membrane depolarization was previously reported in eukaryotic cells, and this capacity was linked to the eosinophil cytotoxic properties during inflammation processes (*14*). However, we must also consider that the cytoplasmic membrane depolarization is not always a determinant killing event. Although it can contribute to the rapid killing of a significant fraction of the bacteria cells, the survival subpopulations can resume their growth (*19*). An additional action either directly at the inner membrane level or toward an intracellular target is sometimes required for an optimal antibiotic activity. Mammalian defensins do display a biphasic killing curve with a rapid, initial decrease and a later slower decrease, suggesting two separate killing mechanisms (*19*). In fact, the innate host defense peptides are characterized for their diverse functions and targets (*21*). A mechanical action at the cell envelope is sometimes accompanied with an additional activity directed against an intracellular target.

Because ECP belongs to the mammalian secretion RNase family, the contribution of its RNase catalytic activity on cellular RNA cannot be totally disregarded. Therefore, together with its action both at the wall and cytoplasmic membrane levels, an additional potential intracellular RNA target should also be considered. The RNase A family includes together with ECP, also named as RNase 3, other RNases with cytotoxic and antipathogen properties, involved in the host defense immunity system (*1, 30, 82–84*). They all share the RNase A superfamily common three-dimensional fold and the main catalytic residues required for the phosphodiester bond cleavage. Although the ribonuclease activity is conserved within all of the family members, its contribution on the antimicrobial capacities is still controversial. In fact, no direct contribution of the RNase catalytic activity to the antimicrobial RNase properties has been identified to date. Some of the RNase A family members have been reported to easily destabilize the lipid bilayers (*15, 17, 32, 85*). Although the RNase catalytic activity is reported not to contribute to the bactericidal capacity for all of the thus far studied members, such as ECP, RNase 7, or the chicken RNase A-2 close homologue (*9, 32, 86*), a functional role *in vivo* cannot be disregarded. It has been proposed that the "RNase A family" backbone might merely serve as a scaffold to support the evolution of non-enzymatic new properties (*86*). However, catalytic activity is also not compulsory for other antimicrobial proteins, such as lysozyme or serprocidins (*87*). Therefore, even if, when assayed *in vitro*, the non-enzymatic proteins display antimicrobial capacity, their catalytic activity may contribute to the bactericidal function *in vivo* (*13*). In fact, innate immunity proteins are often characterized for a wide spectra of action and a multiple target of action. We must also consider that each protein may act in concert with many other immune defense molecules, and complex interactions are likely to occur (*13*). Searching for the common structural and functional determinants of the mammalian RNases involved in the host defense immunity is still a pending challenge.

The studies on the mammalian antimicrobial proteins that participate in the innate immune system are of great interest for the treatment of infection and inflammatory disorders (*88*). The innate immune system is an ancestral common system that the host presents as a first barrier when fighting against invading pathogens. Any disfunction on the innate immunity also has effects on the adaptive immune system. Moreover, many autoimmune diseases are triggered by the innate immunity disregulation. Because the innate immune response is mainly triggered by unique pathogen features, the understanding of the specific interactions of antimicrobial proteins to the bacteria envelope elements is determinant to unravel their bactericidal mechanism.

Moreover, the use of bacteriolysis-inducing agents as antibiotics is considered to be counterproductive in some

cases, because the bacteriolysis results in a massive release of pro-inflammatory cell-wall components. On the contrary, anti-LPS drugs might also serve to prevent the activation of some inflammatory pathways (*52, 56*).

The results on ECP-binding capacity to LPSs and PGNs would help to identify the structural determinants for its specific interaction capacity. We are carrying further analysis to spot the ECP-binding domains to bacteria-unique patterns.

Concluding Remarks. The search for optimization strategies for antimicrobial proteins and peptides is gaining interest because of increased antibiotic resistance. Cationic polypeptides provide an useful working template for a new generation of antimicrobials for the treatment of infections and the modulation of the innate immune system (*89–92*). The importance of the action of potential antibiotics at the bacteria cell wall level has frequently been disregarded. However, experimental evidence indicates that the antimicrobial protein and peptide mechanism of action is highly dependent upon this first encounter step (*93*). We have proven that ECP has a high-affinity binding to LPS and PGN wall components, suggesting that the protein association to the bacteria surface is a determinant initial event in its antimicrobial mechanism.

ACKNOWLEDGMENT

SEM and TEM were performed at the Servei de Microscopia of the Universitat Autònoma de Barcelona (UAB). We thank Francisca Cardoso, Francesc Bohils, and Alex Sánchez for their assistance in electron micrography samples. Spectrofluorescence assays were followed at the Laboratori d'Anàlisi I Fotodocumentació, UAB.

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BI702065B