Articles

Both Aromatic and Cationic Residues Contribute to the Membrane-Lytic and Bactericidal Activity of Eosinophil Cationic Protein†

Esther Carreras,‡ Ester Boix,‡ Helene F. Rosenberg,§ Claudi M. Cuchillo,‡ and M. Victòria Nogués*,‡

Departament de Bioquímica i Biologia Molecular, Facultat de Ciències, Universitat Autònoma de Barcelona, 08193-Bellaterra, Spain, and Laboratory of Host Defenses, NIAID, National Institutes of Health, Bethesda, Maryland 20892 USA

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ABSTRACT: Eosinophil cationic protein (ECP) and eosinophil derived neurotoxin (EDN) are proteins of the ribonuclease A (RNase A) superfamily that have developed biological properties related to the function of eosinophils. ECP is a potent cytotoxic molecule, and although the mechanism is still unknown this cytotoxic activity has been associated with its highly cationic character. Using liposome vesicles as a model, we have demonstrated that ECP tends to disrupt preferentially acidic membranes. On the basis of structure analysis, ECP variants modified at basic and hydrophobic residues have been constructed. Changes in the leakage of liposome vesicles by these ECP variants have indicated the role of both aromatic and basic specific amino acids in cellular membrane disruption. This is the case with the two tryptophans at positions 10 and 35, but not phenylalanine 76, and the two arginines 101 and 104. The bactericidal activity of both native ECP and point-mutated variants, tested against *Escherichia coli* and *Staphylococcus aureus*, suggests that basic amino acids play, in addition to the effect on the disruption of the cellular membrane, other roles such as specific binding on the surface of the bacteria cell.

Eosinophil cationic protein (ECP)[1] is a secretory protein found in the large specific granules of eosinophilic leukocytes. Mature ECP is a single polypeptide with a molecular mass of 15.5 kDa, although several glycosylated forms with molecular masses ranging from 16 to 22 kDa have also been observed. It shares sequence homology with proteins of the RNase A superfamily, a vertebrate-specific enzyme family (*1*), that includes eight human proteins, with different RNase activities, expression patterns, and physiological functions that, in some cases, remain unknown (*2*). Eosinophils also contain another member of the RNase A superfamily, the eosinophil derived neurotoxin (EDN), that shows a 67% amino acid sequence identity with ECP. ECP shares the overall three-dimensional structure and active site amino acids required for RNase activity (*3*), although its catalytic activity is much lower (*4*, *5*). ECP has developed biological properties, related to the functions of the eosinophils, and the ECP levels in different biological fluids correlate with the eosinophil activity which is used as an inflammatory marker (*6*). ECP is a potent cytotoxic molecule, with bactericidal (*7*, *8*) and helminthotoxic (*9*, *10*) properties in contrast with EDN which has very little antibacterial and antiparasitic activities. The cytotoxic effect of ECP can also damage the host epithelial cells (*11*, *12*). Both ECP and EDN are neurotoxic and provoke the Gordon phenomenon when injected intraventricularly to guinea pig and rabbits (*13*); they also possess antiviral activity against the single-stranded RNA respiratory syncytial virus (RSV) (*14*).

It has been suggested that the pathogenic activity of ECP appeared due to a strong positive Darwinian selection occurring after the duplication of the ancestral EDN gene about 30 million years ago, resulting in an increase of the net positive charge of the protein (*15*, *16*). ECP shows an isoelectric point of 10.8, and its highly cationic character is dependent on the number of arginine residues at the molecular surface which may increase the capacity to bind negatively charged molecules of cellular membranes. The

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* To whom correspondence should be addressed. Phone: 34-93-5811256. Fax: 34-93-5811264. E-mail: victoria.nogues@uab.es.

‡ Universitat Autònoma de Barcelona.

§ National Institutes of Health.

[1] Abbreviations: ECP, eosinophil cationic protein; rECP, recombinant eosinophil cationic protein; wtECP, wild-type eosinophil cationic protein; EDN, eosinophil derived neurotoxin; RSV, respiratory syncytial virus; MBP, major basic protein; (Δ115−122)-ECP, an ECP variant in which the region of the loop D115−Y122 has been deleted; (115−122EDN)-ECP, an ECP variant in which the region of the loop D115−Y122 has been substituted by the corresponding sequence of EDN; $(Up)_4U>p$, an oligouridylic acid of five residues ending in a 2′,3′-cyclic phosphate; poly(U), polyuridylic acid; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid disodium salt; DPX, *p*-xylene-bis-pyridinium bromide; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)]; SASA, solvent accessible surface area; CFU, colony forming units; rmsd, residue mean standard deviation.

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mechanism of cytotoxicity of ECP is still unknown and its relation to the RNase activity is controversial. While the ability of ECP to kill bacteria and parasites does not depend on its ribonucleolytic activity (*8*, *17*), ECP and EDN need the catalytic activity to display their neurotoxicity (*18*) and antiviral activity (*19*). Purified ECP is able to form stable nonion selective channels in cell membranes (*20*). Thus, it is plausible to think that ECP can kill cells by a mechanism similar to that of some other pore-forming proteins such as polymerized C9 (*21*). In addition to the transmembrane pore formation, ECP could penetrate the cell where, like other cytotoxic RNases, the subsequent cleavage of RNA would result eventually in cell death. In these cases, reaching the cell's cytosol is very likely the rate limiting step for its cytotoxic activity. It was shown that the increase of net positive charge of RNase A by chemical modification increases its cytotoxicity probably by enhancing its cellular uptake (*22*). On the other hand, ECP blocks selectively the growth of some mammalian cell lines (*23*). Recently, a new member of the RNase superfamily, named RNase 7, with a high cationicity and a broad antimicrobial spectrum, has been identified in human skin and other tissues (*24*, *25*).

The aim of this work is focused on the amino acid residues of ECP, which may interact with the cell membrane and, hence, be responsible for its toxicity. We have used site-directed mutagenesis to modify some residues and checked the cytotoxicity of the ECP variants obtained. On the basis of the three-dimensional structure of ECP (*3*), we have chosen some cationic and hydrophobic residues from the surface of the protein. In addition, we have studied the role of the loop region 115−122, which is specific for both eosinophil RNases and for RNases 6, 7, and 8 and is one of the regions where ECP and EDN differ the most (Figure 1 A). For each ECP variant, the kinetic parameters for ribonucleolytic activity were measured and compared with those of ECP. In addition to the bactericidal effect on both Gram-negative and Gram-positive bacteria, we have determined the ability of wild-type ECP and ECP variants to disrupt the membranes using liposome vesicles as a model.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis, Expression, and Purification of ECP Variants. All mutants were obtained from a human ECP synthetic gene (*5*). R121A- and W10K-ECP mutants and the construction with the deletion of the region 115−122 ((Δ115−122)-ECP) were constructed using standard PCR techniques (*26*). The double variants R75A/F76A-, R121A/Y122A-, W35A/R36A-, R101A/R104A-ECP, as well as the construction where the 115−122 region of ECP is replaced by the corresponding EDN sequence ((115−122EDN)-ECP), were constructed using the Quick Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). All constructs were confirmed by DNA sequencing.

Protein expression in the *Escherichia coli* BL21(DE3) strain (Novagen, Madison, WI) as well as the initial purification steps and folding of the proteins from inclusion bodies were carried out as previously described (*5*). In the subsequent cationic exchange chromatography (Resource S and Mono S columns, Amersham Pharmacia Biotech) purification steps, the proteins were injected in 0.15 M sodium acetate, pH 5, and eluted with a linear NaCl gradient from 0 to 2 M in the same buffer. Differences in the elution pattern were a consequence of the different net charge of each mutant. The homogeneity of the purified proteins was checked with 15% SDS−PAGE and Coomasie Blue staining and MALDI-TOF mass spectrometry (*5*).

Assay of RNase Activity. The RNase activity of the recombinant proteins was determined using the oligouridylic acid $(Up)_4U>p$ as substrate. This substrate was chosen based on the previous kinetic characterization studies of ECP and was prepared from poly(U) digestion (*5*). Assays were carried out in 50 mM MES−NaOH, pH 6.2, at 25 °C, and the kinetic parameters were determined by the spectrophotometric method using 1-cm path length cells (*5*). Substrate concentration was determined using the extinction coefficient $\epsilon_{260} = 12\,500\ M^{-1}\ cm^{-1}$ (*5*). The activity was measured by following the initial reaction velocities using the difference molar absorbance coefficient, in relation to the cleaved phosphodiester bond ($\Delta\epsilon_{280} = 700\ M^{-1}\ cm^{-1}$). Substrate concentrations ranged from 0.01 to 0.3 mM. Final enzyme concentrations varied between 0.14 and 0.5 μM, depending on the activity of each mutant.

Liposome Leakage as a Measure of Membrane Disruption Capacity. Liposome vesicles were prepared by vacuum-drying of a 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC)/1,2 dioleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (DOPG) (3:2 molar ratio) chloroform solution. DOPC and DOPG were from Avanti Polar Lipids. Alternatively, pure DOPG and DOPC vesicles were prepared. To obtain large unilamelar vesicles with incorporated ANTS/DPX, the lipid film was hydrated in 12.5 mM ANTS, 45 mM DPX, 20 mM NaCl, 10 mM Tris, pH 7.5 and mixed overnight in the dark. The suspension was frozen and thawed 20 times in liquid nitrogen. The liposome vesicles were extruded successively through 0.8, 0.4, and 0.1-μm (pore diameter) polycarbonate membranes (Whatman) and centrifuged at 12 000*g* for 10 min to discard nonincorporated lipids. Unencapsulated material was separated from the vesicles by gel filtration on Sephadex G-25 (Amersham Pharmacia Biotech) using 10 mM Tris-HCl, pH 7.5 containing 0.1 M NaCl and 1 mM EDTA as elution buffer. The lipid concentration was determined by a colorimetric assay method for free and phosphorylated glyceric acids (*27*).

Leakage was measured using the 8-aminonaphthalene-1,3,6-trisulfonic acid disodium salt (ANTS)/*p*-xylene-bis-pyridinium bromide (DPX) fluorescence assay (*28*) as described by de los Rios et al. (*29*) with minor modifications. ANTS and DPX were from Molecular Probes. Leakage was measured by fluorescence (Perkin-Elmer 650-4 Fluorescence spectrophotometer). Excitation and emission wavelengths were 386 and 535 nm, respectively. The slit widths were 5 nm for the excitation beam and 10 nm for the emission beam. The percentage of leakage (%*L*) produced by the proteins after 1 h of incubation with the liposomes was calculated with the following equation:

$$(\%L) = 100(F_p - F_o)/(F_{100} - F_o)$$

where F_p is the final fluorescence intensity after addition of the protein (1 h), F_o and F_{100} are the fluorescence intensities before addition of the protein and after addition of 0.5% Triton X100 (Sigma), respectively. Statistical analysis was performed by using the Student's t test. A p value < 0.05 was considered statistically significant.

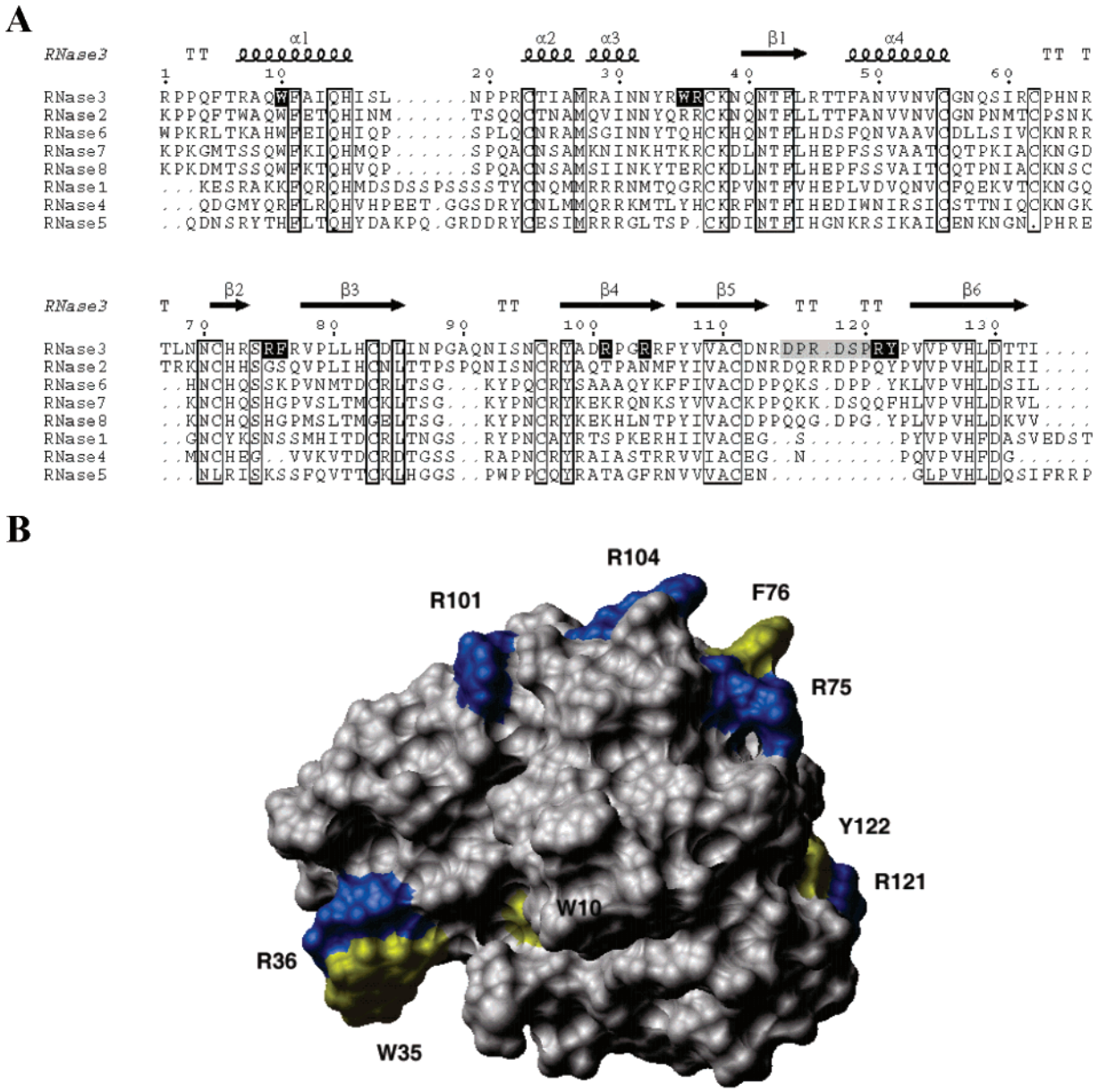

FIGURE 1: Location of the mutated residues on the ECP structure. (A) Primary sequence alignment of human members of the RNase A superfamily. The conserved amino acids are boxed. The selected residues for amino acid substitutions are highlighted in black for single mutation and gray for the loop region. The secondary elements of the ECP structure are shown. The alignment was generated with Clustal W and the picture was drawn using the ESPript program (*43*). (B) Molecular surface representation of the X-ray three-dimensional structure of ECP (1QMT.pdb) showing the position of the mutated residues, with basic amino acid residues (Arg) in blue and hydrophobic residues (Phe, Tyr, and Trp) in yellow. The picture has been drawn using ICM software.

Antibacterial Activity Assay. Antibacterial assay with recombinant proteins was carried out essentially as described by Lehrer et al. (*7*). Overnight cultures of *Escherichia coli* BL21 DE3 strain and *Staphylococcus aureus* 502 A strain (ATCC, Rockville, MD) were washed twice and suspended at 1:100 or 1:1000 with respect to the original volume in 10 mM sodium phosphate, pH 7.5. Aliquots of 20 μL of bacterial suspension were incubated for 4 h at 37 °C with different concentrations (0.5, 1, and 2 μM) of each ECP variant in phosphate buffer or with the same volume of phosphate buffer as a control. The 10-fold dilutions of the treated bacterial suspensions were plated on Luria-Bertani (LB) agar followed by overnight growth at 37 °C, and the colony forming units (CFU)/mL for each treatment was determined. In each experiment, all the assays were carried out in triplicate, and the results obtained were the average of three independent experiments. EDN antibacterial activity was also tested as a negative control. Statistical analysis was performed by using the Student's *t* test. A p value < 0.05 was considered statistically significant. Results were explained in terms of the highest concentration with a significant p value.

The antibacterial activity of recombinant ECP (rECP) at different time intervals between 0 and 4 h was checked on the *E. coli* BL21 DE3 strain by the same procedure using a 2 μM protein concentration.

Molecular Modeling. Three-dimensional models of ECP mutants were obtained based on the X-ray structure of ECP (1QMT·pdb and ref *3*) using the software Internal Coordinate Modeling (ICM, and ref *30*). The solvent accessible surface area (SASA) was calculated using the program MolMol (*31*).

Table 1: Determination of the Total Atomic Charge and Solvent Accessible Surface Area (SASA) Values of Wild-Type and ECP Variants

protein	total atomic charge[a]	% of solvent accessible surface area[b]	total solvent accessible surface area[c]
wild-type ECP	13.73		8196.28
W10K	14.73	9	8207.81
W35A + R36A	12.74	62 + 39	8040.42
R75A + F76A	12.74	53 + 53	8112.36
R101A + R104A	11.75	34 + 47	8098.32
R121A	12.74	56	8016.71
R121A + Y122A	12.74	56 + 20	8118.93
(115−122 EDN)-ECP	13.73		8453.70
(Δ115−122)-ECP	13.75		7857.94

[a] Total atomic charge calculated using the Modeling Software ICM. The electrostatic solvation energy was calculated using the boundary element method (MolSoft and ref *30*). [b] Percent of solvent accessible surface area (SASA) calculated by the MolMol program (*31*), using a solvent radius = 1.4 Å. The values were calculated for the mutated residue in the context of the ECP wild-type surface. Values 0−20% are characteristic of buried residues, 20−40% of partially buried residues, and >40% of surface exposed residues. [c] Total SASA of rECP and the predicted three-dimensional structure of ECP variants calculated using the Modeling Software ICM (MolSoft and ref *30*).

RESULTS

Design and Purification of ECP Mutants. ECP variants were constructed to analyze the contribution of specific basic and aromatic amino acid residues located on the surface of the protein (W10K-, W35A/R36A-, R75A/F76A-, and R101A/R104A-ECP) and at the loop D115−Y122 to the ECP cytotoxic activities (Figure 1). The sequence D115−Y122 of ECP corresponds to a unique exposed region which is a large insertion loop in eosinophil-associated RNases (ECP and EDN) and in RNases 6, 7, and 8. To analyze the role of this region, the following constructions were obtained: the (Δ115−122)-ECP form in which the whole loop was deleted, the (115−122 EDN)-ECP form in which the ECP specific amino acids were substituted by the corresponding EDN residues (116Q/P117R/S120P/R122Q-ECP) and specific variants (R121A-ECP and R121A/Y122-ECP).

The predicted three-dimensional structures of ECP variants using the Internal Coordinate Modeling (ICM, MolSoft) (*31*) did not show any significant deviation from the overall structure of wild-type ECP. The overall residue mean standard deviation (rmsd) of superimposed main chain backbones was in all cases lower than 0.5 Å, except for the form with the EDN loop ((115−122 EDN) ECP), where the loop region has a rmsd around 1.5 Å, and the protein with the deletion of the region D115−Y122 ((Δ115−122)-ECP), where the last five C-terminal residues positions show a shift of about 1.5 Å. All the mutated residues are solvent exposed at the molecule surface, except for W10, which is partly buried and close to the active site cavity. The solvent accessible surface area and the electrostatic potential of the protein show variations depending on the amino acids substitutions (Table 1).

ECP variants obtained by site-directed mutagenesis were purified according to the general procedure described for rECP (*5*), but the steepness of the salt gradient applied to the cation exchange chromatography was optimized in each case. The molecular mass of the purified ECP variants was checked by MALDI-TOF mass spectrometry.

Table 2: Steady-State Kinetic Parameters for the Cleavage of $(Up)_4U>p$ by Wild-Type and ECP Variants[a]

protein	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})
wild-type ECP	0.68 ± 0.06	0.08 ± 0.01	8500
W10K	0.28 ± 0.03	0.07 ± 0.01	4000
W35A/R36A	1.23 ± 0.10	0.09 ± 0.01	13 700
R75A/F76A	0.31 ± 0.01	0.08 ± 0.01	3900
R101A/R104A	1.54 ± 0.14	0.08 ± 0.01	19 200
R121A	1.00 ± 0.09	0.11 ± 0.02	9000
R121A/Y122A	1.07 ± 0.10	0.10 ± 0.02	10 700
(115−122 EDN)-ECP	1.09 ± 0.10	0.13 ± 0.02	8400
(Δ115−122)-ECP	0.34 ± 0.03	0.06 ± 0.01	5700

[a] The substrate was prepared from poly(U) digestion (*5*). The spectrophotometric method was used. Reaction conditions: 50 mM MES−NaOH, pH 6.2 at 25 °C. Substrate concentration ranges were from 0.01 to 0.3 mM. Final enzyme concentrations were from 0.14 to 0.5 μM depending on the activity of each mutant.

Kinetic Characterization of ECP Variants. Table 2 shows the steady-state kinetic parameters for the cleavage of $(Up)_4U>p$ by rECP and the modified forms. This low molecular weight substrate was chosen according to the previous kinetic characterization of rECP (*5*), which indicated an increase in efficiency for the cleavage of oligouridylic acids $(Up)_nU>p$ from $n = 1$ to $n = 4$. ECP has a RNase activity lower than other members of the RNase A superfamily such as EDN or RNase A and the ECP modified forms of this study showed only slight changes in the kinetic parameters with respect to rECP. The highest increase in the catalytic efficiency is observed for the R101A/R104A variant. This effect may be explained in terms of the role of the positive charges located at the surface of the protein as attractants for the negative groups of the substrate as proposed by Mallorquí-Fernández et al. (*32*). The presence of the positive charges might hinder the access of the substrate to the active site or produce a modification of the active site through an indirect effect.

Leakage Capacity on Liposome Vesicles. Lehrer et al. (*7*) analyzed the bactericidal activity of ECP against *E. coli* and *S. aureus* and its effect on the integrity of the bacterial outer and inner membranes of *E. coli*. To further understand the molecular basis of the ECP bactericidal activity (*7*, *8*), we have analyzed the membrane disruptive capacity of the ECP variants with the aim of clarifying the relationship between the cytotoxic activity of ECP and the effect on membrane stability. Lipid vesicles have proven to be a good model to analyze the ECP effect on cellular membranes. Using DOPC/DOPG (3:2 molar ratio) vesicles, a 1.5 μM concentration of ECP produces a leakage of 50% of the total, while the 100% value is obtained at 5 μM (Figure 2A). These protein concentrations correspond to a vesicle/protein molar ratio of 23 and 7, respectively. No detectable effect was observed under the same conditions for both EDN and RNase A with protein concentrations up to 5 μM. These results demonstrate the specific effect of ECP on the membrane's stability. We also analyzed the effect of the net charge of the lipid vesicles on the membrane permeability produced by ECP (Figure 2B) and showed that this effect depends on the presence of negative charges on the synthetic membranes. These results suggest that ECP cytotoxicity may be mediated by the disruption of the cell membrane integrity and that the electrostatic interactions between the negative charges of the membranes and specific positive charges on the protein surface are crucial for its membrane disruptive capacity.

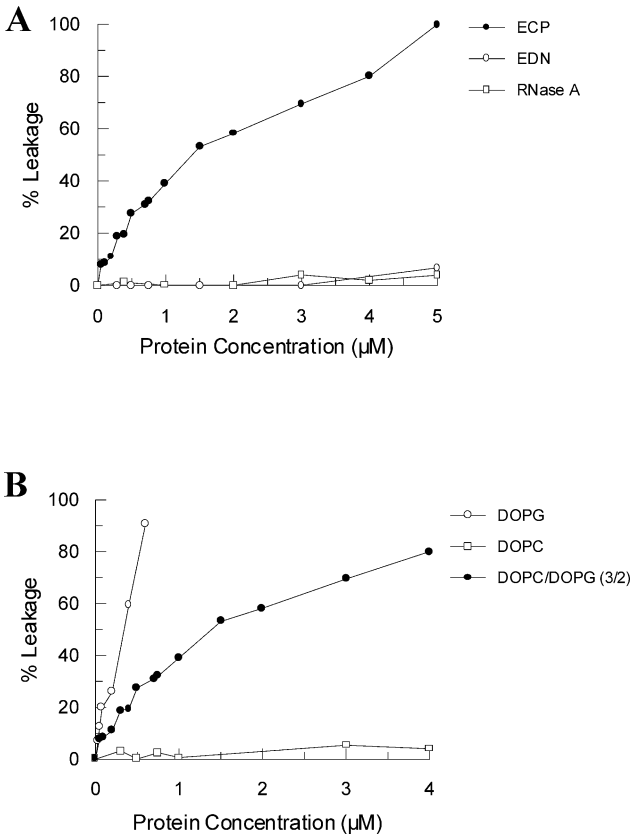

FIGURE 2: Effect of ECP, EDN, and RNase A in the leakage capacity on liposome vesicles. (A) Effect of ECP, EDN, and RNase A on the ANTS/DPX leakage from DOPC and DOPG (3:2 molar ratio) vesicles. (B) Leakage induced by ECP on vesicles containing different proportions of DOPC/DOPG. Only DOPG (○), DOPC/DOPG (3:2 molar ratio) (●) and only DOPC (□).

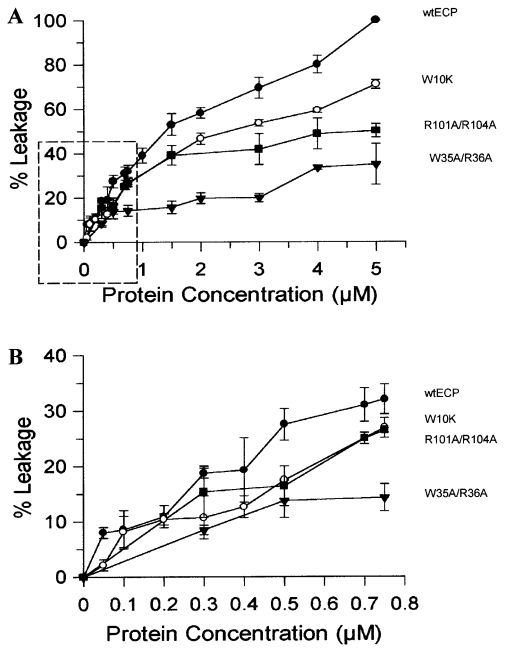

FIGURE 3: Effect of ECP variants which show a significant decrease in the leakage capacity on liposome vesicles compared to the effect of wild-type. (A) Percentage of leakage of W10K-, W35A/R36A-, and R101A/R104A- and wtECP. The effect was analyzed in the leakage of DOPC/DOPG (3/2 molar ratio) vesicles. Statistical significance of the decrease in the percentage of leakage with respect to ECP: $p < 0.01$ for W35A/R36A-ECP, $p < 0.05$ for W10K-ECP (in both cases for all protein concentrations tested) and $p < 0.05$ for R101A/R104A-ECP (for most protein concentrations). (B) Detail of the boxed region in panel A.

We analyzed therefore the effect of the ECP variants modified at specific basic and hydrophobic amino acid residues on the leakage capacity of liposome vesicles. Our results indicate that the observed effect is dependent on the specific amino acid location and not only on the overall net charge or the hydrophobic character of the variant. W35A/R36A-, R101A/R104A-, and W10K-ECP are the variants that cause a significant decrease on the leakage capacity of the liposome vesicles (Figure 3). The effect observed in the W10K-ECP variant demonstrates the role of hydrophobic amino acids without removal of any cationic residue. On the other hand, not every cationic residue is equally important: the leakage is modified in the R101A/R104A variant but not from alterations in R121 or R75. Statistical significance for the decrease in the percentage of leakage of mutants with respect to ECP was $p < 0.01$ for W35A/R36A-ECP, $p < 0.05$ for W10K-ECP (in both cases for all protein concentrations tested), and $p < 0.05$ for R101A/R104A-ECP (for most of protein concentrations). No changes in leakage with respect to ECP was observed for the other mutants (results not shown).

Bactericidal Activity of ECP and ECP Variants. The bactericidal activity of ECP against *E. coli* and *S. aureus* and the effect of different factors as incubation time, protein concentration, temperature, and pH was described by Lehrer et al. (*7*). Figure 4 A shows the kinetics of bactericidal activity of rECP against *E. coli*. The profile of CFU/mL versus incubation time is similar to that of native ECP purified from eosinophils (*7*). This profile indicates that the ECP effect is of a bactericidal nature, although a simultaneous bacteriostatic activity cannot be discarded. Figure 4B,C shows the effect in the bactericidal activity of wild-type and variants of ECP against Gram-negative (*E. coli* BL21 DE3) and Gram-positive (*S. aureus* 502A) strains, expressed as CFU/mL remaining after exposure to different protein concentrations. ECP concentrations up to 2 μM reduce significantly the CFU/mL values of both *E. coli* and *S. aureus*, as previously reported Lehrer et al. (*7*) and Rosenberg (*8*). EDN concentrations up to 2 μM were also tested. No effect was observed (data not shown) in agreement with previous results (*8*).

Figure 4B shows the toxicity of ECP variants with changes in the region corresponding to the loop 115−122 compared to the control. This loop is specific for both human eosinophil RNases and RNases 6, 7, and 8 (Figure 1A) (*2*). A decrease in the CFU/mL value is observed for the variants (Δ115−122)-ECP ($p < 0.02$), R121A- and R12A/Y122A-ECP in Gram-positive bacteria. These variants show a similar effect to that of rECP on the tested Gram-negative bacteria. Figure 4C includes the effect produced by modified forms of ECP which alter specific amino acid residues located on the surface of the protein. W35A/R36A-ECP substitutions decrease the CFU/mL value of ECP for both Gram-negative ($p < 0.02$) and Gram-positive ($p < 0.01$) bacteria. Selective toxicity is found for the other variants studied. W10K-ECP decreases the cytotoxicity of ECP against *S. aureus* ($p < 0.001$), while R101A/R104A-ECP ($p < 0.05$) and R75A/F76A-ECP ($p < 0.01$) reduce the toxicity against *E. coli*. Comparison of Figures 3 and 4 indicates that the bactericidal

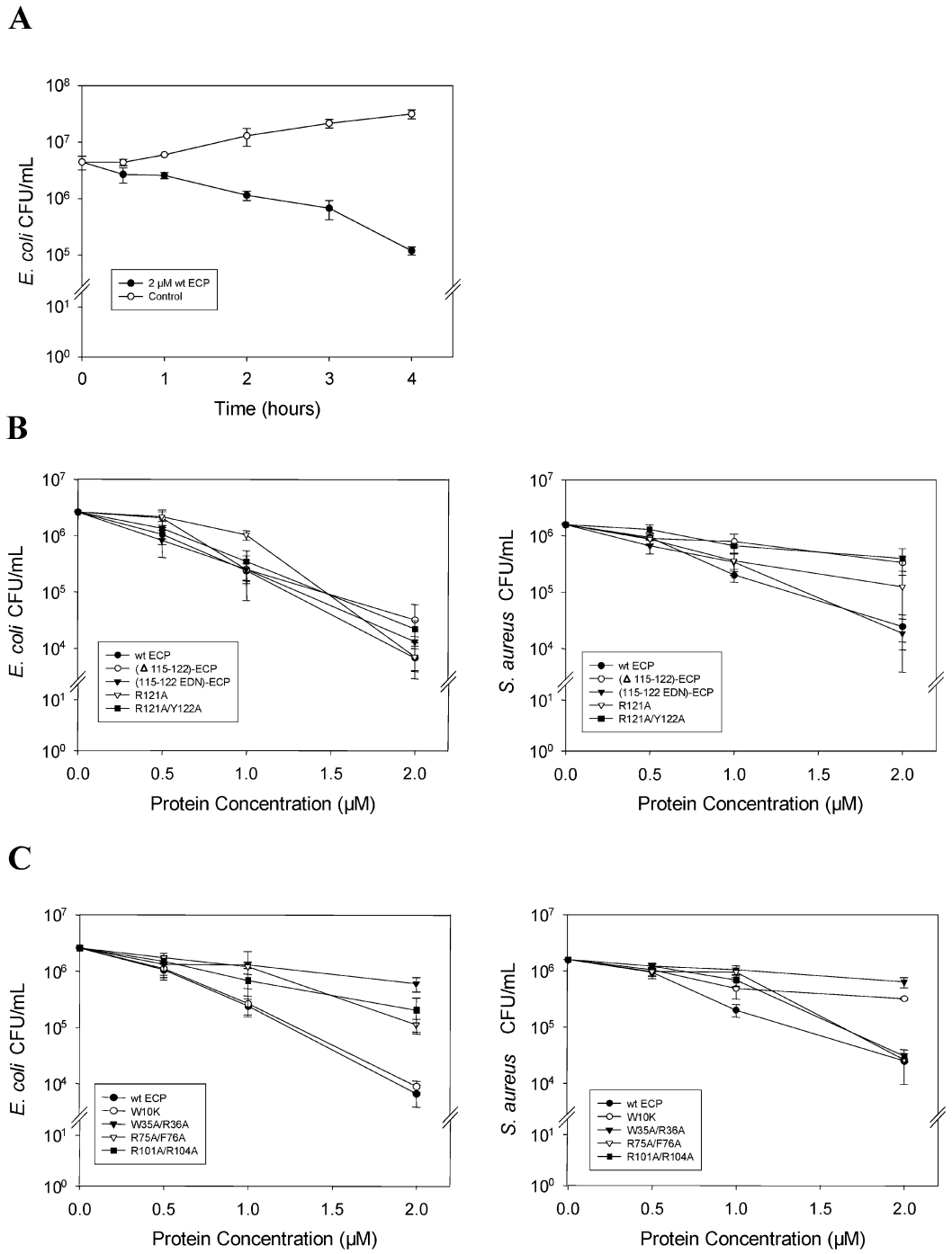

FIGURE 4: Bactericidal activity of wild-type and ECP variants against *S. aureus* and *E. coli*, expressed as percentage of CFU/mL remaining after exposure to different protein concentrations. (A) Kinetics of bactericidal activity of rECP (2 μM) against *E. coli*. (B) Effect of amino acid residues located at the loop D115−Y122. (C) Effect of changes in amino acid residues located on the protein surface. *S. aureus* or *E. coli* cultures in stationary phase were incubated with 0.5, 1, and 2 μM concentration of specific ECP samples or with the same volume of buffer control for 5 h at 37 °C. Statistical significance: $p < 0.02$ for W35A/R36A-, $p < 0.05$ for R101/R104-, and $p < 0.01$ for R75A/F76A-ECP for the bactericidal activity against *E. coli* with respect to ECP and $p < 0.01$ for W35A/R36A-, $p < 0.001$ for W10K-, and $p < 0.02$ for (Δ115−122)-ECP against *S. aureus*.

activity does not correlate well with the leakage capacity on liposome vesicles. The relationship between the bactericidal activity, the structural characteristics, and possible functions of the modified amino acids of each variant is analyzed in the discussion section.

DISCUSSION

It had been proposed that the bactericidal activity of ECP, which is not observed in other proteins of the RNase superfamily such as EDN or RNase A, is a consequence of the high number of arginine residues on the molecular surface (*3*). The overall surface charge for ECP is 14, in contrast to the values of 7 and 3 for EDN and RNase A, respectively (*3*). However, a potential role for hydrophobic residues cannot be excluded. We have constructed mutants that modify specific basic and aromatic amino acid residues located in the D115−Y122 loop and on the surface of the protein with the aim of analyzing the molecular basis of this

activity. The variants were selected from comparison of the sequence and three-dimensional superposition of both ECP and EDN. Residues only present in the ECP structure were considered as potentially important for the bactericidal activity (Figure 1A). We also centered the analysis on the regions with the highest potential for interaction with the cell membrane and on the most exposed positive and hydrophobic residues on the surface of the protein (Figure 1B). Therefore, we constructed variants that modify the loop 115−122, by deletion of the sequence 115−122 ((Δ115−122)-ECP form), by substitution of ECP specific amino acids for the corresponding EDN residues (−116Q/P117R/S120P/R122Q-ECP) ((115−122 EDN)-ECP form) and specific variants of this region (R121A-, R121A/Y122-ECP). Variants that alter the hydrophobic and/or basic character of the protein surface as W10K-, W35A/R36A-, R75A/F76A-, and R101A/R104A-ECP were also constructed. ECP has its only two tryptophan residues at positions 10 and 35. W35 is specific for ECP in the RNase A superfamily and in the ECP structure is the most exposed surface residue, and its change to alanine modifies considerably the solvent accessible surface area (Table 1). In the W35A/R36A-ECP variant, the adjacent arginine residue (R36) was also changed to alanine to eliminate the corresponding contribution of the electrostatic interaction of the region. Tryptophan in position 10 is specific for eosinophil RNases and is less exposed to the solvent than W35 (Figure 1B). We changed tryptophan 10 to lysine to mimic an RNase key residue (K7) involved both in substrate binding and catalytic activity (*33*). Finally, the substituted basic amino acids (R75, R101, and R104) have their side chain exposed to the solvent and, together with F76, form a cluster involved in the crystal packing of ECP (*3*).

ECP shows a low RNase activity in comparison with EDN or RNase A, and previous studies by Rosenberg (*8*) showed that the ECP bactericidal activity was maintained in mutants where the RNase activity had been abolished. Our results indicate that the amino acid changes introduced in our variants do not modify substantially the RNase kinetic parameters (Table 2) except for the R101A/R104A and W35A/R36A variants that showed an increase in activity. However, in the two latter cases, a decrease in the bactericidal activity against *E. coli* (Figure 4) and in the leakage capacity on liposome vesicles (Figure 3) were also observed. These results suggest that the changes in the RNase activity are not directly correlated with the bactericidal activity. A recent study on the cationization of both RNase A and human RNase 1 has concluded that the increase in the protein net charge facilitates their cellular uptake. In that case, contrary to the case of ECP, catalytic activity is required for the cytotoxicity to malignant cell lines (*22*).

We have demonstrated that ECP, in contrast to EDN and RNase A, is able to permeate liposome vesicles, and that the net charge of the lipid vesicles is crucial for its action (Figure 2). Besides, differences dependent on the analyzed bacterial strains suggest that selective structural features of the protein are involved in the binding and disruption capacities of ECP to particular bacterial cell walls and membranes.

While mutants W35A/R36A-, R101A/R104A-, and W10K-ECP have shown a decrease in the bactericidal activity, at least in one of the tested bacterial strains, in parallel with the decrease in the leakage of liposome vesicles with respect to wild-type (Figure 3), R75A/F76A- and (Δ115−122)-ECP mutants show a reduced bactericidal activity which does not correlate with a decrease in leakage activity. Studies of antimicrobial peptides indicate that tryptophan prefers lipid−water interfaces, while phenylalanine, with its nonpolar aromatic ring, is mostly located in the core of the membrane (*34*−*36*). Our results point out the importance of specific aromatic residues in the process: tryptophan, but not phenylalanine substitution, reduces liposome leakage. The effect on the decrease of leakage of liposome vesicles in the case of the R101A/R104A- variant may be associated with the strong electrostatic interaction that may take place in the wild-type ECP (wtECP) between the positive charges in the side chains of these very close arginine residues and the negative groups of DOPG lipids. Our results indicate that ECP destabilizes preferentially acidic liposome vesicles (Figure 2). Major basic protein (MBP), another eosinophil granule protein with a very high isoelectric point (pI $\approx$ 11), which contains alternating hydrophobic and cationic residues, mainly interacts with anionic lipid vesicles, inducing their aggregation and lysis (*37*). Dathe et al. (*38*) have analyzed the destabilization of liposome vesicles by peptides and have observed that the most active peptides against negatively charged bilayers, such as we have demonstrated for ECP, accumulate on the surface of the membrane without deep insertion into the acyl chain region because of electrostatic binding in the lipid headgroup region.

The bactericidal activity of proteins may be due to a particular binding or other specific effect on the surface of the bacterial cell, depending on whether it is a Gram-positive or Gram-negative species. The polypeptides may bind to Gram-negative bacteria through electrostatic interactions with the negatively charged lipopolysaccharide, the major component of the outer leaflet of the outer membrane, followed by insertion into the lipid matrix. After crossing the peptidoglycan layer, the protein, ECP in this case, could bind to the negatively charged groups of the cytoplasmic membrane, bring about its destabilization through hydrophobic interactions, and translocate across the bilayer (*38*). Lehrer et al. (*7*) have demonstrated that ECP and MBP produce a decrease in the colony count in parallel with the permeabilization of the outer and inner membranes of *E. coli*. In the case of Gram-positive bacteria, the negative charge on the surface is provided by the teichoic acids of the cell wall. Before interacting with the negatively charged cytoplasmic membrane, the protein should have to cross first the thick peptidoglycan barrier, and then, the effects on the cytoplasmic membrane might be similar to those described for Gram-negative bacteria. In both cases, two types of specific interactions are necessary: on one hand, the electrostatic binding between the negative groups of the membrane and basic amino acids of the protein and, on the other hand, specific hydrophobic interactions involved in membrane disruption. However, the hydrophobic and electrostatic binding energies of proteins at the membrane interfaces are not just additive; in fact, the hydrophobic free energy reduces the effective valence of cationic peptides (*39*). In this context, the studied amino acids may play different roles in the ECP bactericidal activity. Those changes that only modify the level of bactericidal activity but do not cause liposome vesicle leakage (R75A/F76A- and (Δ115−122)-ECP) could

correspond to residues involved in the binding processes between the protein and the bacteria. In contrast, amino acid substitutions that alter liposome vesicles leakage may be associated with effects on membrane destabilization (W10K-, W35A/R36A-, R101A/R104A-ECP). No clear relationship between membrane disruption and bactericidal activity has also been reported in the case of many cationic antimicrobial peptides (*40*, *41*).

In conclusion, the studied amino acids play different roles in the bactericidal activity of ECP. The modified residues in the variants that selectively modify the level of bactericidal activity or the liposome vesicle leakage, may be involved in the different steps of the bactericidal process. We are planning to further characterize the ECP binding to bacteria and the membrane disruption process, as well as other potential steps in the protein bactericidal mechanism. Bactericidal proteins and peptides constitute a first immune defense barrier (*42*). The understanding of the molecular basis of the antimicrobial activity of proteins and peptides involved in the host immune defense offers an alternative strategy to antibiotic development.

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