

Lysyl-Phosphatidylglycerol Attenuates Membrane Perturbation Rather than Surface Association of the Cationic Antimicrobial Peptide 6W-RP-1 in a Model Membrane System: Implications for Daptomycin Resistance[∇]

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The presence of the cationic phospholipid lysyl-phosphatidylglycerol (lysyl-PG) in staphylococcal cytoplasmic membranes has been linked to increased resistance to cationic compounds, including antibiotics such as daptomycin as well as host defense antimicrobial peptides. We investigated the effects of lysyl-PG on binding of 6W-RP-1, a synthetic antimicrobial peptide, to lipid vesicles and on peptide-induced membrane permeabilization. Unexpectedly, physiological lysyl-PG concentrations only minimally reduced membrane binding of 6W-RP-1. In contrast, 6W-RP-1-induced dye leakage was severely inhibited by lysyl-PG, suggesting that lysyl-PG primarily impacts membrane defect formation.

Aminoacylated phosphatidylglycerols are common lipids in the cytoplasmic membranes of Gram-positive bacteria, most notably in the genera *Staphylococcus*, *Bacillus*, *Clostridium*, *Lactobacillus*, *Listeria*, and *Streptococcus* (7, 11, 15). Esterification of the phosphatidylglycerol (PG) headgroup occurs with either lysine (lysyl-PG), alanine (alanyl-PG), or, less commonly, ornithine (ornithyl-PG) or arginine (arginyl-PG). Such modifications may serve to modulate the cytoplasmic membrane charge, as the adduct converts the anionic PG into a net cationic or zwitterionic lipid, depending on the amino acid involved (13). In *Staphylococcus aureus*, this reaction is mediated by the multiple peptide resistance factor *F* (MprF), an integral membrane protein that catalyzes the transfer of the aminoacyl group from Lys-tRNA^{Lys} to the *sn*-3 position of the PG headgroup (14).

In *S. aureus*, resistance to killing by mammalian cationic antimicrobial peptides (CAPs) has been attributed to reduced peptide binding to the bacterial cell surface (16). Furthermore, resistance to daptomycin, a calcium-dependent, cationic lipopeptide, has been suggested to have coevolved with reduced susceptibility to host defense CAPs (8, 9). The presence of lysyl-PG in the bacterial membrane has been clearly linked to these phenomena in *S. aureus* and *Listeria* (8, 17). For instance, reduced levels of lysyl-PG were found to increase the susceptibility of *S. aureus* to many antibiotics, including some CAPs (10, 18, 19), and mutant *S. aureus* strains that do not produce lysyl-PG exhibit reduced virulence in a variety of *in vivo* models (18). Of note, the observed correlation between PG lysinylation and reduced susceptibility to cationic antibiotics (e.g., daptomycin) and CAPs has been attributed to a relative de-

crease in the negative surface charge of the target bacterial cytoplasmic membrane as a consequence of PG modification (8, 10, 12, 13, 18). However, no detailed analyses of the mechanism(s) by which lysyl-PG protects the organism from cationic agent-induced killing have been carried out in either whole bacterial cells or model membranes. For this reason, we employed model membrane systems composed of various PG-to-lysyl-PG ratios and the synthetic peptide 6W-RP-1 to further probe this interaction. 6W-RP-1 is a fluorescent derivative of RP-1, a synthetic peptide modeled in part on the C-terminal helical and microbicidal domain of the mammalian platelet factor 4 (PF-4) family of molecules (20).

The peptide 6W-RP-1 (ALYKKWKKKLLKSLKRLG), a tryptophan-substituted congener of the parent peptide, RP-1 (20), was synthesized and authenticated as described previously (20). Large unilamellar vesicles (LUVs) were prepared by mixing chloroform solutions of lipids in the appropriate proportions (1). To mimic the cytoplasmic membrane of *S. aureus*, a 70:30 mixture of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (POPG) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was used instead of pure POPG to increase vesicle stability. The impact of lysyl-DOPG on peptide activity was investigated by replacing various fractions of POPG with 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(3-lysyl(1-glycerol))] (lysyl-DOPG). For lipid vesicles used in the binding studies, the fluorescent lipid 7MC-POPE (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine [POPE], derivatized with 7MC through an amide bond to the amino group of the ethanolamine headgroup) was synthesized as previously described (5) and added in a chloroform solution to the unlabeled lipids.

The association kinetics of 6W-RP-1 with lipid vesicles were measured by Förster resonance energy transfer (FRET) from the Trp residue in the peptide to the acceptor fluorophore, 7MC-POPE, embedded in the bilayer at a concentration of 2 mol% (5). Peptide binding leads to an increase in FRET and

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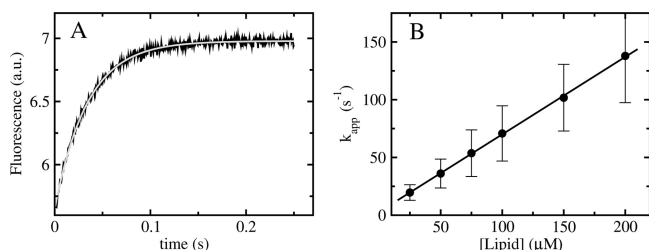
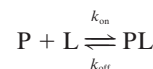


FIG. 1. Binding of 6W-RP-1 to vesicles composed of POPS and POPC (70:30 ratio). (A) Experimental binding kinetics recorded using 50 μM lipid and 0.5 μM peptide. The experimental data are the averages of five individual traces to improve the signal-to-noise ratio; the solid line is a single exponential fit to the data. (B) The apparent rate constant (k_{app}) was plotted against the lipid concentration to obtain k_{on} and k_{off} , as explained in the text. The error bars represent the standard deviations from five sets of experiments.

can thus be monitored through the increased fluorescence emission from the acceptor. The reaction was followed in a stopped-flow fluorimeter (SX.18MV; Applied Photophysics) as previously described (1). The peptide concentration was held constant at 0.5 μM in all FRET experiments, and the lipid concentration was varied between 25 and 200 μM .

If the initial peptide binding to lipid vesicles is well separated in time from other bilayer-perturbing events, peptide binding is described by the simple equilibrium equation



where P stands for free peptide in solution, L represents lipid, PL represents lipid-bound peptides, and k_{on} and k_{off} are the rate constants for peptide association and dissociation, respectively (1). The coupled differential equations that describe this equilibrium are $d[P]/dt = -k_{\text{on}} [L] [P] + k_{\text{off}} [PL]$ $d[PL]/dt = k_{\text{on}} [L] [P] - k_{\text{off}} [PL]$.

As the lipid concentration does not change throughout the experiment, the product of k_{on} and $[L]$ remains constant. Hence, the kinetics of peptide binding follow a pseudo-first-order rate law and are characterized by an apparent rate constant, k_{app} , equal to $(k_{\text{on}} [L] + k_{\text{off}})$, which can be obtained from a fit of the experimental traces to an exponential function (Fig. 1A). Since the magnitude of k_{app} depends on the lipid concentration, a plot of k_{app} against $[L]$ will be linear (Fig. 1B). The molecular rate constants of peptide binding, k_{on} values, were obtained from the slope of a linear regression to the data points and k_{off} from the y intercept. The dissociation constant, K_D , was then obtained from the $k_{\text{off}}/k_{\text{on}}$ ratio.

We determined k_{app} as a function of lysyl-DOPG content and found that at a given lipid concentration, k_{app} varied only slightly with a lysyl-DOPG content between 0 and 30 mol% but decreased significantly at lysyl-DOPG concentrations exceeding ~ 30 mol% (Fig. 2A). By using the dependence of k_{app} on

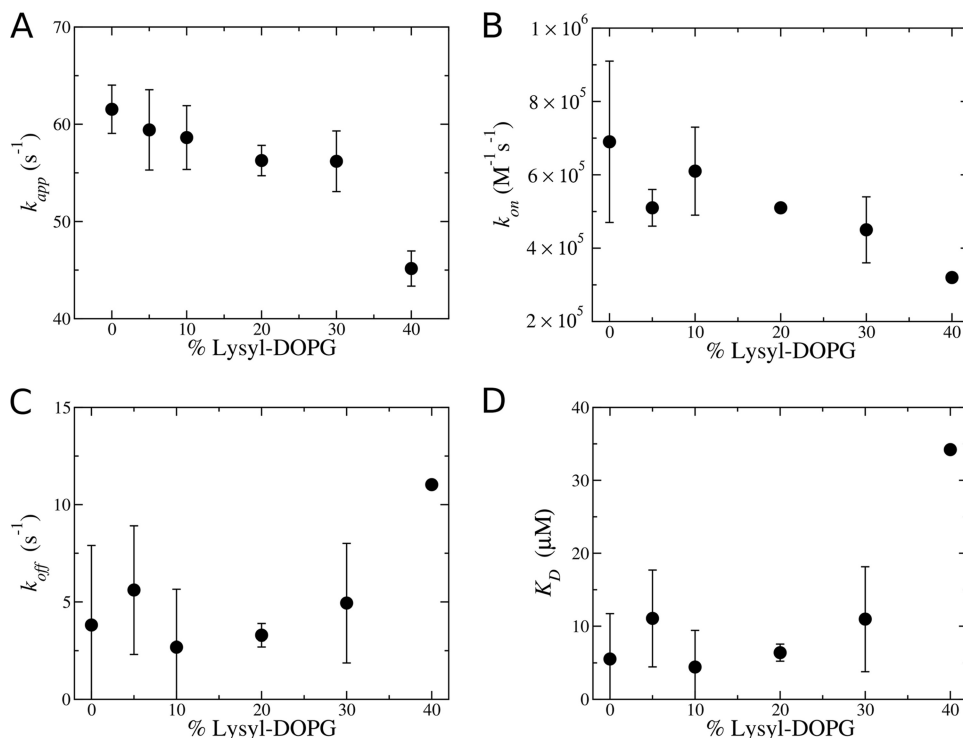


FIG. 2. 6W-RP-1 interaction with lipid vesicles as a function of the lysyl-DOPG content. The vesicle composition is based on a POPS:POPC ratio of 70:30, in which a fraction of the POPS was replaced by lysyl-DOPG. (A) The apparent rate constant, k_{app} , as a function of lysyl-PG content, measured at a lipid concentration of 100 μM . (B to D) The molecular rate constant for peptide binding, k_{on} (B), the molecular rate constant for 6W-RP-1 desorption, k_{off} (C), and the dissociation constant, K_D (D). The peptide concentration was 0.5 μM in all experiments. The error bars are the standard deviations for a minimum of two and usually three sets of experiments.

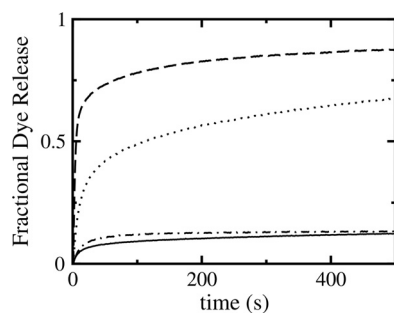


FIG. 3. Carboxyfluorescein leakage induced by 6W-RP-1 (5 μ M) from vesicles (50 μ M lipid) composed of POPG:POPC:lysyl-DOPG (70:30:0; dashed line), POPG:POPC:lysyl-DOPG (50:30:20; dotted line), POPG:POPC:lysyl-DOPG (40:30:30; dash-dotted line), and POPG:POPC:lysyl-DOPG (30:30:40; solid line). All curves are normalized to the maximum dye release, which was determined by adding Triton X-100 to the vesicles at a final concentration of 1% to dissolve the vesicles (1).

lipid concentration to obtain the molecular rate constants k_{on} and k_{off} for each lysyl-DOPG concentration, we were able to show that this effect is caused primarily by a change in k_{off} . The rate constant for 6W-RP-1 binding to lipid vesicles varied linearly with lysyl-DOPG content (Fig. 2B). However, the dependence was relatively weak, dropping to about 60% of its original value at 40% lysyl-DOPG. In contrast, k_{off} was found to remain essentially constant up to an overall lysyl-DOPG content of \sim 30 mol% (Fig. 2C), which encompasses the lysinylation profile of most clinical *S. aureus* isolates (6, 12, 13). Only at a lysyl-DOPG concentration of $>$ 30 mol% did k_{off} become notably faster, leading to a significantly reduced net association and a correspondingly higher K_D (Fig. 2D). We propose that this observed lack of correlation between the lysyl-DOPG concentration and peptide binding until the lysyl-DOPG content exceeds 30 mol% is related to peptide-induced lipid domain formation that results in the separation of anionic PG from the cationic lysyl-DOPG and the remaining membrane lipids. In a homogeneous bilayer, the Gibbs free energy (ΔG°) of binding can be expected to be largely a linear function of the lysyl-DOPG fraction. In that case, we would predict the dissociation constant, K_D , to depend exponentially on the fraction of lysyl-DOPG in the mixture, according to the equation $K_D = \exp(\Delta G^\circ/RT)$, which it clearly did not in our studies (Fig. 2D).

In contrast to the data on 6W-RP-1 binding, the extent of peptide-induced membrane permeabilization of lipid vesicles was strongly inhibited as a function of lysyl-DOPG content, even at low lysyl-DOPG concentrations. Dye leakage was assessed by encapsulating a self-quenching concentration of carboxyfluorescein (CF) in LUVs by methods described previously (1). A 5 μ M peptide solution was allowed to interact with a lipid suspension, and the rate and extent of dye leakage were monitored. The kinetics of CF leakage, measured by the relief of fluorescence self-quenching, were recorded in a stopped-flow fluorimeter to ensure rapid and complete mixing of the reactants. We found that inclusion of 20 mol% lysyl-DOPG decreased both the extent and rate of dye leakage, and at 30 mol%, at which peptide binding is still largely unaffected, dye leakage was essentially abolished (Fig. 3). Although unanticipated, this finding is consistent with a biological function of

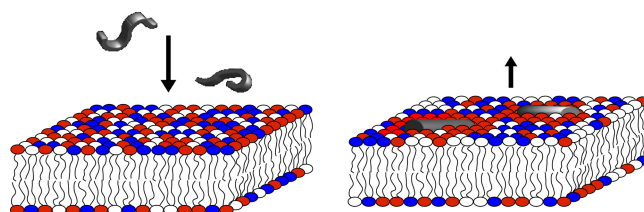


FIG. 4. Cartoon showing peptide binding to a homogeneous bilayer (left) and desorption from a bilayer with PG domains induced by 6W-RP-1 binding (right). Red headgroups represent unmodified PG; blue headgroups represent lysyl-PG; white headgroups represent PC. In the left panel, peptides are drawn as gray, random structures above the bilayer, representing the unfolded state in solution; in the right panel, they are shown as gray rods to indicate their more ordered conformation in association with the bilayer-bound state.

lysyl-DOPG that derives in part from the inhibition of membrane perturbation and leakage, rather than the inhibition of CAP binding. Thus, while daptomycin-resistant clinical *S. aureus* strains often exhibit increased lysyl-PG membrane content and an enhanced surface positive charge (8), the current findings suggest that such phenotypes are more likely to stabilize membrane integrity than repel cationic antimicrobial agents.

Figure 4 represents a working model that interprets our experimental findings. In this view, binding of 6W-RP-1 occurs from an unstructured state in solution to a bilayer in which the three lipid types (PG, lysyl-PG, and PC) are homogeneously distributed. The preferential interaction of the peptide with anionic PG (Fig. 4, red lipids) leads to the formation of lipid domains enriched in PG (Fig. 4, right) and the exclusion of cationic lysyl-PG (Fig. 4, blue lipids). We postulate that at a high lysyl-PG content, the remaining fraction of PG becomes limiting and the peptides no longer bind efficiently. It is not clear why this process *per se* should lead to an inhibition of dye leakage from vesicles; it is conceivable that the presence of the positively charged lysyl-PG leads to an overall stabilization of a membrane rich in anionic PG. The possibility of PG domain formation in mixed lipid vesicles induced by the binding of a cationic peptide has been suggested previously (2–4).

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