Human Peptidoglycan Recognition Proteins Require Zinc to Kill Both Gram-Positive and Gram-Negative Bacteria and Are Synergistic with Antibacterial Peptides

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Human Peptidoglycan Recognition Proteins Require Zinc to Kill Both Gram-Positive and Gram-Negative Bacteria and Are Synergistic with Antibacterial Peptides

Minhui Wang, Li-Hui Liu, Shiyong Wang, Xiaofeng Lu, Dipika Gupta, and Roman Dziarski

Mammals have four peptidoglycan recognition proteins (PGRPs or PGLYRPs), which are secreted innate immunity pattern recognition molecules with effector functions. In this study, we demonstrate that human PGLYRP-1, PGLYRP-3, PGLYRP-4, and PGLYRP-3:4 have Zn$^{2+}$-dependent bactericidal activity against both Gram-positive and Gram-negative bacteria at physiologic Zn$^{2+}$ concentrations found in serum, sweat, saliva, and other body fluids. The requirement for Zn$^{2+}$ can only be partially replaced by Ca$^{2+}$ for killing of Gram-positive bacteria but not for killing of Gram-negative bacteria. The bactericidal activity of PGLYRPs is salt insensitive and requires N-glycosylation of PGLYRPs. The LD$_{50}$ of PGLYRPs for Gram-positive and Gram-negative bacteria is 0.3–1.7 μM, and killing of bacteria by PGLYRPs, in contrast to killing by antibacterial peptides, does not involve permeabilization of cytoplasmic membrane. PGLYRPs and antibacterial peptides (phospholipase A2, group IIA phospholipase A2, and Roman Dziarski) synergistically kill Gram-positive and Gram-negative bacteria. These results demonstrate that PGLYRPs are a novel class of recognition and effector molecules with broad Zn$^{2+}$-dependent bactericidal activity against both Gram-positive and Gram-negative bacteria that are synergistic with antibacterial peptides. The Journal of Immunology, 2007, 178: 3116–3125.

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3 Abbreviations used in this paper: PGRP or PGLYRP, peptidoglycan recognition proteins; BPI, bactericidal permeability-increasing protein; HBD-3, human β-defensin-3; HNP-1, human neutrophil protein-1 (α-defensin); LTA, lipoteichoic acid; MurNAc, N-acetylmuramoyl-L-alanine amidase; PBPs, penicillin-binding proteins; PGN, peptidoglycan; PMNs, polymorphonuclear leukocytes; PRRs, pattern recognition receptors; Pseudomonas aeruginosa; R. c证书; S. aureus; T. mentagrophytes; V. vulnificus.

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Gram-positive bacteria is Ca\(^{2+}\) dependent (10) and because other divalent cations present in body secre
tions and tissue fluids influence the activity of other antimicrobial proteins (16, 17, 27–40), in this study, we tested the hypothesis that other divalent cations may also play an important role in bactericidal activity of human PGLYRPs. Moreover, because PGLYRPs are secreted into body fluids that also contain many antibacterial peptides with different bactericidal mechanisms from PGLYRPs (10), we also tested the hypothesis that PGLYRPs may kill bacteria synergistically with these peptides. We discovered that PGLYRPs have higher bacteri
cidal activity against Gram-positive bacteria and become highly bactericidal for Gram-negative bacteria in the presence Zn\(^{2+}\) and also that PGLYRPs kill bacteria synergistically together with membrane-damaging antibacterial peptides.

Materials and Methods

Materials

Human PGLYRPs-1, PGLYRPs-3, PGLYRPs-4, and PGLYRPs-3:4 were ex
pressed in S2 cells and purified as previously described (10), except that instead of Ca\(^{2+}\): 40 \(\mu\)M Zn\(^{2+}\) (for testing on Gram-negative bacteria) or 2 mM Ca\(^{2+}\) and 10 \(\mu\)M Cu\(^{2+}\) (for testing on Gram-positive bacteria) were used in protein purification buffers, and 10 \(\mu\)M Zn\(^{2+}\) (for Gram-negative bacteria) or 2 mM Ca\(^{2+}\), 10 \(\mu\)M Zn\(^{2+}\), and 10 \(\mu\)M Cu\(^{2+}\) (for Gram-positive bacteria) were used in the final dialysis buffer. Where
ever indicated, other combinations of divalent cations were also used in some experiments in the purification and dialysis buffers as described in Results. For Zn\(^{2+}\) removal, proteins were incubated with 100 \(\mu\)M EGTA (10). De-N-glycosylation was performed as described previously (10).

Human group IIA phospholipase A\(_2\) (PLA\(_2\)) (41) was amplified from the Image clone ID 152802 (obtained from Open Biosystems) by PCR using forward (GGCATCTAATTTGGTGAATTTCCACAGATG) and re
class (AACTGGACAGCAGGAGGGCTGCTCCCTCTC) primers and subcloned into the BglII and EcoRI sites of the pMT/BiP/V5-His expression vector (Invitrogen Technology). To generate human PLA\(_2\)/PGLYRP-1 fusion protein, the PGLYRP-1 coding region (10) was amplified by PCR using forward (GTGGATTCGCTCAGGAGACAGAAGACCCGG) and reverse (CATCTAGAGGGGAGCGGTGAGTGCAAT) primers and was inserted 3\’ of the PLA\(_2\) into the EcoRI and XbaI sites. Human Fc IgG1 fragment was amplified from the clone ID 5480266 (obtained from Amer
casian Type Culture Collection) by PCR using forward (CAGAGATCTGG CCGCAATCTTGAGCAAACCT) and reverse (ATACCGGTATT TCCATGTCCGGTGTCCCTG) primers (codons 239–250) and subcloned into the BglII and AgeI sites of the pMT/BiP/V5-His expression vector. To generate human PGLYRP-1/Fc fusion protein, the Fc coding region was amplified as above with forward (CGTCTAGAGACAGCAAGGCTGGCTCCTC) primer and the same reverse primer and was inserted 3\’ of the PGLYRP-1 (10) into the XbaI and AgeI sites. All constructs were confirmed by restriction digestion and by sequencing. The proteins were expressed and purified from S2 cell supernatants as described above for PGLYRPs. Each of these purified proteins (10 \(\mu\)g/\(\mu\)l) yielded one band on Coomassie brilliant blue-stained gel of comparable purity to PGLYRPs (10).

Bacteria

The following bacteria were used: Bacillus subtilis ATCC 6533, Enterococcus faecalis ATCC 19433, Escherichia coli K12 (used in most experiments), E. coli O18:K1:H7:Bort (42), Lactobacillus acidophilus ATCC 4356, Listeria monocytogenes ATCC 19115, Micrococcus luteus ATCC 4698, Proteus vulgaris ATCC 13315, Pseudomonas aeruginosa ATCC 27853, Salmonella enterica ATCC 6539, Shigella sonnei ATCC 25931, Staphylococcus aureus RB (10), and Streptococcus pyogenes ATCC 49399.

Antibacterial assays

Bactericidal and bacteriostatic activity of human PGLYRPs and antibac
terial peptides was assayed on logarithmically growing bacteria at 0.4–
1.2 \(\times\) 10\(^{8}\)/ml as previously described (10) with recombinant human Fc IgG1 fragment as a control protein or PGLYRPs or other test proteins at the concentrations indicated in Results. The standard medium was 5 mM Tris (pH 7.6), with 150 mM NaCl, 5 \(\mu\)M ZnSO\(_4\), 5% glycerol, and 1% Luria-
Bertani (LB) for Gram-negative bacteria, or the same medium plus 1 mM CaCl\(_2\) and 5 \(\mu\)M CuSO\(_4\) and either 1% LB or 1% BHI for Gram-positive bacteria (10), unless otherwise indicated (i.e., in some experiments com
bining various divalent cations listed in Table 1 were used, as indicated in Results). The numbers of bacteria were determined by colony counts on LB or BHI agar plates (10). In some experiments, as indicated in Results, 0.525 M sucrose was included in the assay and dilution medium, and bacteria were overlaid in 0.7% agar onto 1.5% agar LB plates, both containing 0.5 M sucrose (10). All other controls were performed as de
cribed previously (10). Bactericidal activity is defined as an at least 2 log\(_{10}\) decrease in the number of inoculated bacteria in 6 h; bacteriostatic activity is defined as an inhibition of growth of bacteria or a decrease in the number of inoculated bacteria of <2 log\(_{10}\) in 6 h. The results are means of three (mostly) or two experiments; the average SEs were 18% of the mean (ranged from 6 to 28%) and are not shown because the error bars were mostly smaller than the size of the symbols in the figures. The significance of differences was calculated using the Student’s t test, and all differences in bacteri
cial numbers >1 log\(_{10}\) were statistically significant at p < 0.05 (and most often at p < 0.01).

Table 1. Concentrations of divalent cations in human sweat and saliva and in PGLYRP bactericidal assay buffer

<table>
<thead>
<tr>
<th>Cation</th>
<th>Sweat</th>
<th>Saliva</th>
<th>Assay Buffers*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(^{2+})</td>
<td>0.5 mM</td>
<td>2 mM</td>
<td>1 mM</td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>100 (\mu)M</td>
<td>40 (\mu)M</td>
<td>50 (\mu)M</td>
</tr>
<tr>
<td>Zn(^{2+})</td>
<td>17 (\mu)M</td>
<td>2 (\mu)M</td>
<td>5 (\mu)M</td>
</tr>
<tr>
<td>Fe(^{2+})</td>
<td>6 (\mu)M</td>
<td>6 (\mu)M</td>
<td>5 (\mu)M</td>
</tr>
<tr>
<td>Cu(^{2+})</td>
<td>5 (\mu)M</td>
<td>5 (\mu)M</td>
<td>5 (\mu)M</td>
</tr>
<tr>
<td>Se(^{2+})</td>
<td>1 (\mu)M</td>
<td>39 (\mu)M</td>
<td></td>
</tr>
<tr>
<td>Ni(^{2+})</td>
<td>0.8 (\mu)M</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Mn(^{2+})</td>
<td>0.4 (\mu)M</td>
<td>1 (\mu)M</td>
<td></td>
</tr>
</tbody>
</table>

*Final concentration of ions in the bactericidal assays used individually or in combinations as indicated in Figs. 1 and 2. Two times higher concentrations were used for protein purification and storage buffers, and other components were used as described in Materials and Methods.

Results

Bactericidal activity of PGLYRPs against Gram-positive bacteria is enhanced by Zn\(^{2+}\)

We previously demonstrated that human PGLYRPs were strongly bactericidal for some pathogenic Gram-positive bacteria (S. aureus and L. monocytogenes) and nonpathogenic transient Gram-positive bacteria (B. subtilis, Bacillus cereus, Bacillus licheniformis, and L. acidophilus) (10). This bactericidal activity required the presence of Ca\(^{2+}\), which could not be replaced by Mg\(^{2+}\) (10). However, under these conditions, these PGLYRPs were only bacteriostatic (but not bactericidal) for other pathogenic (S. pyogenes) or normal flora (Staphylococcus epidermidis, M. luteus, E. fae
calis, and Streptococcus agalactiae) Gram-positive bacteria and for all Gram-negative bacteria (E. coli, P. vulgaris, and Enterobacter cloacae) (10). However, these PGLYRPs were losing bactericidal activity upon storage at 4°C in our buffer with Ca\(^{2+}\), suggesting that the ion or pH conditions of this buffer were not optimal for the stability and activity of these proteins. Because PGLYRPs are se
creted (PGLYRP-1 into PMN’s granules, PGLYRP-2 into the se
rin, PGLYRP-3, PGLYRP-4, and PGLYRP-3:4 into sweat, and PGLYRP-4 also into saliva) (10), we reasoned that they should be more stable (and maybe more active) under the conditions that
closely mimic these body fluids. To test this hypothesis, we determined the bactericidal activity of PGLYRPs that were purified and assayed in the presence of six divalent cations that are found in sweat and saliva in at least micromole concentrations (Table I) (30).

PGLYRP-4, purified and tested in the presence of six divalent cations (Table I), had higher bactericidal activity for *S. aureus* than PGLYRP-4 purified and tested as before (10) in the presence of Ca$^{2+}$/H$^{+}$ only (Fig. 1A). To determine which of these cations were required for this higher bactericidal activity, we tested bactericidal activity of PGLYRP-4 purified and assayed in the presence of individual cations or various combinations of these cations. Equally high bactericidal activity of PGLYRP-4 for *S. aureus* was observed in the presence of three cations (Ca$^{2+}$/H$^{+}$, Zn$^{2+}$/H$^{+}$, and Cu$^{2+}$/H$^{+}$) and almost as high in the presence of Zn$^{2+}$/H$^{+}$ only (Fig. 1A). These results demonstrate that Zn$^{2+}$/H$^{+}$ is the single most essential cation needed for bactericidal activity of PGLYRP-4 with optimal effect at $\approx 5 \mu$M, which is the approximate concentration of Zn$^{2+}$/H$^{+}$ found in body secretions, such as sweat and saliva (Table I), where PGLYRP-4 is found. PGLYRP-4 at lower concentrations of Zn$^{2+}$/H$^{+}$ was less active (data not shown). Zn$^{2+}$/H$^{+}$ is probably required for the stability or proper conformation of PGLYRP proteins because it had to be present during the entire purification of PGLYRPs and bactericidal assays. The high bactericidal activity of PGLYRPs purified in the absence of Zn$^{2+}$/H$^{+}$ could not be restored by simply adding Zn$^{2+}$/H$^{+}$ to the protein purified without Zn$^{2+}$/H$^{+}$ or to the bactericidal assay (data not shown).

To determine whether other human bactericidal PGLYRPs had the same ion requirements, we tested bactericidal activity of PGLYRP-4 purified and tested as before (10) in the presence of Ca$^{2+}$ only (Fig. 1A). To determine which of these cations were required for this higher bactericidal activity, we tested bactericidal activity of PGLYRP-4 purified and assayed in the presence of individual cations or various combinations of these cations. Equally high bactericidal activity of PGLYRP-4 for *S. aureus* was observed in the presence of three cations (Ca$^{2+}$/H$^{+}$, Zn$^{2+}$/H$^{+}$, and Cu$^{2+}$/H$^{+}$) and almost as high in the presence of Zn$^{2+}$/H$^{+}$ only (Fig. 1A). These results demonstrate that Zn$^{2+}$/H$^{+}$ is the single most essential cation needed for bactericidal activity of PGLYRP-4 with optimal effect at $\approx 5 \mu$M, which is the approximate concentration of Zn$^{2+}$/H$^{+}$ found in body secretions, such as sweat and saliva (Table I), where PGLYRP-4 is found. PGLYRP-4 at lower concentrations of Zn$^{2+}$/H$^{+}$ was less active (data not shown). Zn$^{2+}$/H$^{+}$ is probably required for the stability or proper conformation of PGLYRP proteins because it had to be present during the entire purification of PGLYRPs and bactericidal assays. The high bactericidal activity of PGLYRPs purified in the absence of Zn$^{2+}$/H$^{+}$ could not be restored by simply adding Zn$^{2+}$/H$^{+}$ to the protein purified without Zn$^{2+}$/H$^{+}$ or to the bactericidal assay (data not shown).

To determine whether other human bactericidal PGLYRPs had the same ion requirements, we tested the effect of Zn$^{2+}$/H$^{+}$, Cu$^{2+}$/H$^{+}$, and Fe$^{2+}$/H$^{+}$ (alone and in combinations) on the bactericidal activity of PGLYRP-1, PGLYRP-3, and PGLYRP-3:4 (PGLYRP-3: PGLYRP-4 heterodimer) for *S. aureus*. Zn$^{2+}$/H$^{+}$ was the most essential cation for the bactericidal activity of all human PGLYRPs (Fig. 1, A–D). Note that these assays were performed at lower concentrations of PGLYRPs than before (10).

**FIGURE 1.** Bactericidal activity of PGLYRPs for *S. aureus* and *S. pyogenes* is enhanced by Zn$^{2+}$/H$^{+}$. *S. aureus* or *S. pyogenes* was incubated with 50–100 $\mu$g/ml (*S. aureus*) or 100–150 $\mu$g/ml (*S. pyogenes*) of the indicated PGLYRPs purified and assayed in the presence of the indicated six ions (Ca$^{2+}$/H$^{+}$, Mg$^{2+}$/H$^{+}$, Zn$^{2+}$/H$^{+}$, Fe$^{2+}$/H$^{+}$, Cu$^{2+}$/H$^{+}$, and Mn$^{2+}$/H$^{+}$) individually or in combination (as indicated on the figure) at the concentrations shown in Table I or Fc IgG fragment as a control. The numbers of bacteria were determined by colony counts. The results are means of two to three experiments.

**FIGURE 2.** PGLYRPs are bactericidal for *E. coli* in the presence of Zn$^{2+}$/H$^{+}$. *E. coli* was incubated with 100–150 $\mu$g/ml of the indicated PGLYRPs purified and assayed in the presence of the indicated ions (Ca$^{2+}$/H$^{+}$, Zn$^{2+}$/H$^{+}$, and Cu$^{2+}$/H$^{+}$) individually or in combination at the concentrations shown in Table I or Fc IgG fragment as a control. The numbers of bacteria were determined by colony counts. The results are means of two to three experiments.
In our previous study, PGLYRPs purified and tested in the presence of Ca\(^{2+}\) (without Zn\(^{2+}\)) were bactericidal for some pathogenic Gram-positive bacteria (S. aureus and L. monocytogenes) and for nonpathogenic transient Gram-positive bacteria (Bacillus sp. and L. acidophilus), but only bacteriostatic for other pathogenic (S. pyogenes) or normal flora Gram-positive bacteria (10). Because PGLYRPs purified and assayed in the presence of Zn\(^{2+}\) were more active against S. aureus, we next tested whether these PGLYRPs were also more active against other Gram-positive bacteria. Indeed, all four PGLYRPs (PGLYRP-1, PGLYRP-3, PGLYRP-4, and PGLYRP3:4) purified and assayed in the presence of Zn\(^{2+}\) instead of Ca\(^{2+}\) also had higher bactericidal activity against L. monocytogenes, B. subtilis, and L. acidophilus (data not shown) and were bactericidal for S. pyogenes at 100–150 \(\mu\)g/ml (Fig. 1E). At 100–150 \(\mu\)g/ml, they were still only bacteriostatic for normal flora Gram-positive bacteria (M. luteus and E. faecalis), and at 300 \(\mu\)g/ml, they were bactericidal for M. luteus but not for E. faecalis (data not shown).

PGLYRP-1 was most stable at pH 4.5, but its bactericidal activity could not be assayed at such a low pH because pH < 6 decreased the viability of S. aureus and other bacteria by itself.

PGLYRP-1 had higher bactericidal activity for S. aureus at pH 6.4 than at neutral or alkaline pH (data not shown). These results are consistent with the location of PGLYRP-1 in PMN’s granules (13–15), which are usually acidic or become acidic following phagocytosis. PGLYRP-3, PGLYRP-4, and PGLYRP3:4 were most stable and most bactericidal for S. aureus and other bacteria at pH 7.6 (data not shown), which falls within the range of pH found in the intestine, saliva, and sweat (30).

Because NaCl inhibits bactericidal activity of many mammalian antibacterial peptides (e.g., defensins) (16, 17), we next tested whether bactericidal activity of PGLYRPs was also salt sensitive. However, bactericidal activity of PGLYRPs for S. aureus and other bacteria was not inhibited by NaCl and was optimal at 100–150 mM NaCl (data not shown), which falls within the range of NaCl concentrations found in the intestine, saliva, sweat, and other body fluids (30).
sensitive or more sensitive than E. coli. Similar high sensitivity was also observed for the highly pathogenic encapsulated invasive strain E. coli O18:K1:H7:Bort (data not shown). The LD$_{99}$ (the concentration that kills 99% of bacteria) for E. coli was 30 μg/ml for PGLYRP-1 (0.68 μM) and PGLYRP-3 (0.34 μM), 70 μg/ml for PGLYRP-3:4 (0.71 μM), and 200 μg/ml for PGLYRP-4 (1.7 μM), compared with ~45 μg/ml for S. aureus for PGLYRP-1 (1.0 μM), PGLYRP-3 (0.50 μM), PGLYRP-3:4 (0.46 μM), and PGLYRP-4 (0.39 μM) (Fig. 4). The LD$_{99}$ for PLA$_{2}$, which is one of the most active antibacterial peptides (18–21), for E. coli and S. aureus were 30 μg/ml (18 μM) and 40 μg/ml (24 μM), respectively.

The requirement for Zn$^{2+}$ was further tested by removal of Zn$^{2+}$ with EGTA, which has a high affinity for Zn$^{2+}$ (the log stability constant for Zn$^{2+}$ is 12.9, compared with 11.0 for Ca$^{2+}$). Treatment of PGLYRPs with EGTA (in a buffer with 5 μM Zn$^{2+}$ only with no other divalent cations) abolished the bactericidal activities of PGLYRPs for Gram-negative bacteria (Fig. 5A), thus confirming the requirement for Zn$^{2+}$ for their bactericidal activity.

N-Glycosylation of all PGLYRPs was also required for their bactericidal effect on Gram-negative bacteria (similar to Gram-positive bacteria; Ref. 10), as treatment of PGLYRPs with N-glycosidase abolished their bactericidal activity (Fig. 5B). Boiling of PGLYRPs for 30 min also abolished the bactericidal activity of all PGLYRPs for Gram-negative bacteria (data not shown).

Bactericidal effect of PGLYRPs on Gram-negative bacteria does not involve permeabilization of cell membrane

We have previously shown that PGLYRPs, in contrast to antibacterial peptides, do not permeabilize membranes in Gram-positive bacteria and kill them by targeting their cell wall (10, 11). Therefore, we next tested whether PGLYRPs kill Gram-negative bacteria by permeabilizing their cell membranes or by targeting their cell walls. We compared the effects of PGLYRPs on Gram-negative bacteria to the effects of membrane-permeabilizing antibacterial peptide (magainin) and an inhibitor of peptidoglycan biosynthesis (ampicillin). We measured membrane permeabilization in real time using a membrane-nonpermeable dye (SYTOX-green, which fluoresces upon binding to DNA, after entering cells with damaged membranes) and compared it with bacterial killing measured by colony counts. Vertebrate antibacterial peptides (e.g., magainin, PLA$_{2}$, and defensins) kill bacteria by traversing bacterial membranes, whereas PGLYRPs do not.

**FIGURE 5.** Bactericidal activity of PGLYRPs for E. coli requires Zn$^{2+}$ and N-glycosylation. E. coli was incubated with the indicated PGLYRPs (100–150 μg/ml PGLYRP-1, PGLYRP-3, or PGLYRP-3:4 or 300 μg/ml PGLYRP-4) or Fc IgG fragment as control (all purified in the presence of Zn$^{2+}$) treated or untreated as indicated with EGTA or N-glycosidase. The numbers of bacteria were determined by colony counts. The results are means of two experiments.

**FIGURE 6.** Bactericidal activity of PGLYRPs for E. coli does not involve permeabilization of cytoplasmic membrane. Membrane permeabilization and killing of E. coli incubated with magainin (200 μg/ml), ampicillin (500 μg/ml), or PGLYRPs (200 μg/ml) were measured in medium without or with 0.5 M sucrose. The results are means of two to three experiments.
cell wall and permeabilizing bacterial cytoplasmic membrane (16, 17). Therefore, the kinetics of membrane permeabilization by antibacterial peptides correlates with the kinetics of bacterial killing and such an expected result was obtained in magainin-treated E. coli (Fig. 6). Similar results were obtained in the medium with 0.5 M sucrose, which protects bacteria from killing by antibiotics that inhibit cell wall synthesis (because bacteria can re-synthesize their cell wall), but not from killing by membrane permeabilization, as confirmed by our results with magainin-treated E. coli (Fig. 6).

Antibiotics (such as ampicillin) target cell wall and kill bacteria by inhibiting peptidoglycan synthesis. However, ampicillin does not cause early permeabilization of bacterial cytoplasmic membranes because ampicillin-treated bacteria only die when they start to grow and are unable to synthesize peptidoglycan. Therefore, in ampicillin-treated bacteria, membrane permeabilization is substantially delayed until the bacteria have grown without synthesizing peptidoglycan, which we also observed in our experiments in ampicillin-treated E. coli (Fig. 6). Also as expected, killing of E. coli by ampicillin and delayed permeabilization of bacterial membranes were prevented in a medium with 0.5 M sucrose (Fig. 6).

PGLYRPs rapidly killed E. coli (within 1 h, as measured by colony counts) but did not permeabilize their cytoplasmic membranes for the entire 6-h observation period (Fig. 6). Killing of E. coli by PGLYRPs was not prevented by 0.5 M sucrose (Fig. 6). These results contrast the ability of hyperosmotic medium with sucrose to prevent killing of Gram-positive bacteria by PGLYRPs, or by peptidoglycan-lytic enzymes, or by penicillin (Fig. 5 in Ref. 10) and killing of E. coli by ampicillin (Fig. 6). These results indicate that the effect of PGLYRPs on Gram-negative bacteria becomes irreversible in <1 h, i.e., from this point on the bacteria are unable to grow (hence, no formation of colonies when bacteria are plated).

These results demonstrate that, in contrast to antibacterial peptides, PGLYRPs do not kill Gram-negative bacteria by permeabilizing their cytoplasmic membranes. These results also indicate that PGLYRPs do not kill bacteria by lysing their cell wall because cell-wall lytic enzymes cause early permeabilization of cytoplasmic membrane due to hypertonic shock and osmotic lysis, which (together with the killing) is preventable in hyperosmotic medium with sucrose (10). By contrast, killing of Gram-negative bacteria by PGLYRPs is not prevented in 0.5 M sucrose (Fig. 6), which resembles the combined synergistic effect of peptidoglycan-lytic enzymes and PGLYRPs on Gram-positive bacteria, whose killing under these conditions is also not prevented by sucrose (10).

**Synergistic killing of Gram-positive and Gram-negative bacteria by PGLYRPs and antibacterial peptides**

Several host defenses usually work together to eliminate bacteria and they are often synergistic. PGLYRP-1 is present in PMNs’ granules (13–15), and PGLYRP-3 and PGLYRP-4 are present on the skin, mucous membranes, and in body secretions (10) together with antibacterial peptides, such as PLA2, defensins, and BPI (16–22). If two antibacterial agents have different bactericidal mechanisms, they are likely to kill bacteria synergistically. Because antibacterial peptides kill bacteria by permeabilizing their membranes (16–22) and PGLYRPs by targeting bacterial cell walls (10, 11), here we tested the hypothesis that PGLYRPs may kill bacteria synergistically with antibacterial peptides.

When S. aureus or L. monocytogenes was incubated with subbactericidal concentrations of PGLYRP-1, PGLYRP-3, or PGLYRP-4 and PLA2 or α-defensin (HNP-1), the killing of these bacteria was enhanced 300–10,000 times, compared with the killing by each compound alone, thus demonstrating a strong synergistic killing of Gram-positive bacteria (Fig. 7, p < 0.001; and Fig. 9, p < 0.01). Similarly, incubation of E. coli with subbactericidal concentrations of PGLYRP-1 or PGLYRP-3 and PLA2, β-defensin (HBD-3), or BPI resulted in 1,000–20,000 times enhancement of bacterial killing compared with the killing by each compound alone, thus also demonstrating a strong synergistic killing of Gram-negative bacteria (Fig. 8, p < 0.001; and Fig. 9, p < 0.01).

In similar experiments, S. aureus was also synergistically killed by subbactericidal concentrations of PGLYRP-3 or PGLYRP-4 and lysostaphin (a peptidoglycan-lytic enzyme) (100–1000 times enhanced killing, data not shown), which further confirms different mechanisms of bacterial killing by PGLYRPs and lysostaphin. Similarly, S. aureus was also synergistically killed by subbactericidal concentrations of penicillin and lysostaphin (100 times enhanced killing, data not shown), confirming that another pair of bactericidal agents with different mechanisms of action also synergistically kills bacteria.

To further test whether synergistic effect of PGLYRPs and antibacterial peptides was due to their action on different parts of the bacterial cell, we constructed a PLA2-PGLYRP-1 fusion protein.
PGLYRP-1 and PLA2 indeed act on different parts of the bacterial cell (cell wall and cytoplasmic membrane). However, if both proteins have the same target (e.g., cell membrane), the fusion protein could still show enhanced activity similar to the two unfused proteins. Our results showed that the PLA2-PGLYRP-1 fusion protein did not have the enhanced bactericidal activity of the unfused PGLYRP-1 and PLA2 added together for S. aureus and E. coli (Fig. 9). As additional controls, we tested PGLYRP-1-Fc fusion protein alone, which had the same bactericidal activity as PGLYRP-1, and PLA2-Fc fusion protein alone, which had the same bactericidal activity as PLA2 (data not shown), demonstrating that fusing PGLYRP-1 or PLA2 with another protein or increasing the size of PGLYRP-1 or PLA2 does not inhibit their bactericidal activities. These results further confirm that PGLYRPs and PLA2 target different parts of the bacterial cell.

In conclusion, our results demonstrate strong synergistic killing of bacteria by PGLYRPs and PLA2, defensins, BPI, or a peptidoglycan-lytic enzyme, and further confirm that PGLYRPs and these antibacterial peptides kill bacteria by different mechanisms.

**Discussion**

We have demonstrated here that bactericidal activity of human PGLYRP-1, PGLYRP-3, PGLYRP-4, and PGLYRP-3:4 for both Gram-positive and Gram-negative bacteria requires Zn2+. For killing of Gram-negative bacteria, Zn2+ cannot be replaced by other cations, but for killing of Gram-positive bacteria Zn2+ can be partially replaced by Ca2+. This Zn2+ dependence explains why in our previous experiments PGLYRPs purified in the presence of Ca2+ (without Zn2+) were not bactericidal for Gram-negative bacteria and were only bactericidal for some Gram-positive bacteria (10).

Physiologic concentrations Zn2+ (~2–5 μM) were sufficient for full bactericidal activity of human PGLYRPs. Zn2+ is present in sweat at ~17 μM, in saliva at ~2 μM, and in plasma at ~15 μM (30). Zn2+ is an essential component of many proteins, such as enzymes and transcription factors, and is required for their structure and function (31). The concentration of extracellular and intracellular Zn2+ is tightly regulated by Zn2+ transporters and Zn2+-binding proteins (31–33). Proper function of host immunity requires Zn2+ because Zn2+-deficient individuals have increased susceptibility to infections and Zn2+ supplementation restores or enhances resistance to infections and the functions of innate and acquired immunity, although the exact mechanisms involved are mostly unknown (34–40). Several peptidoglycan- or polysaccharide-lytic enzymes contain Zn2+ (43–46), and some antimicrobial peptides require Zn2+ for their antimicrobial activity (47–52).

However, the role of Zn2+ and other divalent cations in innate immunity is complex because, in addition to its enhancing effect, Zn2+ can also inhibit the activity of some antimicrobial proteins, especially Ca2+-binding proteins psoriasin (26) and calprotectin (27–29). Although the exact mechanism of antimicrobial activity of psoriasin and calprotectin are unknown, they are thought to be antimicrobial through Zn2+ deprivation because they bind Zn2+, and Zn2+ supplementation reduces their antimicrobial activity (26–29). However, Zn2+ may also inhibit antimicrobial effect of calprotectin by inducing a change in its conformation, rather than by reversing microbial Zn2+ deprivation (27).

Therefore, proper compartmentalization of Zn2+ and antimicrobial proteins may be important. For example, PGLYRP-3 and PGLYRP-4 (which require Zn2+ for activity) are present in sweat glands, salivary glands, and the eye (10), and calprotectin and psoriasin (whose activity is inhibited by Zn2+) are not (26). Moreover, although both calprotectin and PGLYRP-1 are expressed in neutrophils, calprotectin is present in the cytosol and primary and...
secondary granules (53), and PGLYRP-1 is present in the tertiary granules (13). Also, concentrations of Zn²⁺ that enhance or inhibit microbial killing are different: PGLYRPs require 2–5 μM Zn²⁺ for killing bacteria, whereas inhibition of antimicrobial effect of calprotectin and psoriasin requires 10–30 μM Zn²⁺, and therefore, at low physiological Zn²⁺ concentrations, all these proteins are antimicrobial. Thus, proper control of Zn²⁺ concentration is essential for efficient antimicrobial immunity. In this context, it is interesting to note that activation of innate immunity TLRs by bacteria regulates Zn²⁺ homeostasis in dendritic cells and induces net export of Zn²⁺ from the cells (54). This process is essential for the activation of dendritic and other immune cells (54), and it is tempting to speculate that it may also provide extracellular Zn²⁺ for antimicrobial peptides and PGLYRPs. This would be a feasible mechanism because intracellular Zn²⁺ concentration is ~150–300 μM (33), and extracellular PGLYRPs require ~2–5 μM Zn²⁺ for bactericidal activity.

The requirement for Zn²⁺ for bactericidal activity of PGLYRP-1, PGLYRP-3, PGLYRP-4, and PGLYRP-3:4 is likely through its effect on PGLYRPs and not on the bacteria (perhaps to stabilize PGLYRPs) because Zn²⁺ presence is required both during the purification of PGLYRPs and in the bactericidal assay. PGLYRP-2 and other amidase-active PGRPs (e.g., Drosophila PGRP-SC1 and PGRP-LB) have a Zn²⁺-binding site and require Zn²⁺ for their amidase activity (5, 6, 55, 56). These amidase-active PGRPs have four Zn²⁺-binding amino acids in their enzyme active site (5, 6, 55, 56). Other PGRPs, however, do not have amidase activity (such as mammalian PGLYRP-1, PGLYRP-3, and PGLYRP-4), do not have all four Zn²⁺-binding amino acids conserved: they have Ser instead of Cys in the position corresponding to the Zn²⁺-binding Cys530 in human PGLYRP-2 (5, 6). Thus, it is not known where the Zn²⁺-binding site in mammalian PGLYRP-1, PGLYRP-3, and PGLYRP-4 may be. These PGLYRPs will have to be crystallized in the presence of Zn²⁺ to unequivocally determine whether they bind Zn²⁺ and where the Zn²⁺ binding site is located.

Our current and previous results demonstrate that human PGLYRP-1, PGLYRP-3, and PGLYRP-4 are bactericidal proteins with broad bactericidal activity against most Gram-positive and Gram-negative bacteria, both pathogenic and nonpathogenic. Only some normal flora Gram-positive bacteria show partial resistance to bactericidal effects of these PGLYRPs, which is likely an adaptation of these bacteria needed for colonizing the skin, mucous membranes, or the intestine, where PGLYRP-3 and PGLYRP-4 are produced, as discussed previously (10, 11). PGLYRPs are bacteriostatic for these normal flora bacteria, and this bacteriostatic effect may prevent the overgrowth of normal flora bacteria on skin and mucous membranes.

Bactericidal activity of PGLYRP-1, PGLYRP-3, and PGLYRP-4 for both Gram-positive and Gram-negative bacteria is likely to be a general characteristic of these PGLYRPs in all mammals because their sequences are highly conserved, because they show similar patterns of expression, and because a bovine PGLYRP-1 ortholog has been already shown to kill both Gram-positive and Gram-negative bacteria (14, 15). Although Zn²⁺ was not used in the purification or bactericidal assay of bovine PGLYRP-1, this was a native (not recombinant) protein purified from the leukocyte granules, and it probably retained Zn²⁺ that was likely bound to it in vivo. Consistent with our results on human PGLYRP-1, PGLYRP-3, PGLYRP-4, and PGLYRP3:4, bactericidal activity of bovine PGLYRP-1 for Gram-negative bacteria was also inhibited by Ca²⁺ (15).

We have also demonstrated that human PGLYRPs synergistically kill both Gram-positive and Gram-negative bacteria together with antibacterial peptides, such as PLAT and α- and β-defensins, when both compounds are present at subbactericidal concentrations. This synergism is likely to greatly enhance antibacterial defenses in vivo because both PGLYRPs and antibacterial peptides are found in the same locations in the body, such as PMN granules (PGLYRP-1, PLAT₂, defensins, and BPI), skin (PGLYRP-3, PGLYRP-4, and defensins), eyes (PGLYRP-3, PGLYRP-4, and PLAT₂), and oral cavity and intestinal tract (PGLYRP-3, PGLYRP-4, PLAT₂, and defensins) (3, 10–22).

The mechanism of bacterial killing by PGLYRPs seems to be unique and different from other known antibacterial agents. PGLYRPs do not permeabilize cytoplasmic membranes, which is the main mechanism of killing by antibacterial peptides, such as defensins or PLAT₂ (16–22). PGLYRPs also likely do not kill bacteria by hydrolyzing peptidoglycan because peptidoglycan-hydrolytic activity of PGLYRP-1, PGLYRP-3, and PGLYRP-4 could not be demonstrated (6, 10), and because treatment of bacteria with peptidoglycan-lytic enzymes results in osmotic lysis of bacteria, which causes rapid membrane permeabilization that is prevented in hyperosmotic medium (10). Such membrane permeabilization is not observed in bacteria treated with PGLYRPs (Ref. 10 and this article). If PGLYRPs kill bacteria by hydrolysis of peptidoglycan, the rate of hydrolysis would have to be very slow, still not noticeable after 6 h of exposure of bacteria to PGLYRPs. In our experiments, E. coli cell membrane was still not permeabilized after 6 h of incubation with PGLYRPs (Fig. 6), and if peptidoglycan was hydrolyzed, this would have resulted in osmotic lysis of bacteria and membrane permeabilization. Therefore, to kill bacteria, such a slow peptidoglycan hydrolysis would have to be coupled with essentially irreversible binding of PGLYRPs to bacterial cell wall, which would result in delayed, rather than immediate killing of bacteria. Long exposure of bacteria to bovine PGLYRP-1 did result in bacterial lysis, but it was concluded that bacterial lysis was not required and was not responsible for bacterial killing because bacteriolytic activity of bovine PGLYRP-1 was heat stable (15).

Measuring killing by colony counts on plates does not indicate that at the time of bacterial dilution and plating the bacteria are dead; it only indicates that at the time of plating the cidal effect is irreversible—bacteria may die hours later or may be simply unable to divide and form a colony. Many bactericidal antibiotics (β-lactams, aminoglycosides, etc.) have a similar effect—they irreversibly bind to their target, but bacteria actually die later, when they are unable to grow or divide. For this reason, in ampicillin-treated E. coli by 6 h of exposure, the cell membrane was beginning to be permeabilized indicating osmotic lysis of growing bacteria (Fig. 6). The kinetics of killing and membrane permeabilization of Gram-positive bacteria by penicillin and PGLYRPs were similar, and the killing of Gram-positive bacteria by both compounds was prevented in hyperosmotic medium (10). Killing of ampicillin-treated E. coli was prevented in hyperosmotic medium, as expected (Fig. 6), which reflects the ability of E. coli (similar to S. aureus in our previous experiments; Ref. 10) to resynthesize the transpeptidases irreversibly inactivated by ampicillin (after diluting and plating the bacteria in hyperosmotic medium). However, killing of E. coli by PGLYRPs was not prevented in hyperosmotic medium (Fig. 6), similar to the combined killing effect of PGLYRPs and lysostaphin on S. aureus (Fig. 5 in Ref. 10).

Peptidoglycan is a polymer of β-(1-4)-linked N-acetylglucosamine and N-acetylmuramic acid (MurNac) cross-linked by short peptides containing alternating L- and D-amino acids (3). Each PGRP domain has a ligand-binding groove that binds peptidoglycan through its MurNac-tripeptide or MurNac-pentapeptide (56–62). Binding of mammalian PGLYRPs to peptidoglycan is
likely involved in bactericidal activity of these PGLYRPs because exogenously added peptidoglycan inhibits bactericidal activity of PGLYRPs for Gram-positive bacteria (10). However, killing of Gram-negative bacteria by PGLYRPs likely involves an additional step because, in Gram-negative bacteria, peptidoglycan is not exposed on the cell surface since it is located underneath the LPS-containing outer membrane. Killing of Gram-negative bacteria likely involves initial binding of PGLYRPs to the outer membrane because killing of Gram-negative bacteria by bovine PGLYRP-1 (15) and by human PGLYRP-1, PGLYRP-3, and PGLYRP-4 (M. Wang and R. Dziarski, unpublished results) is inhibited by LPS. In Gram-positive bacteria, the initial binding of PGLYRPs to bacteria may involve lipoteichoic acid (LTA), in addition to peptidoglycan, because LTA also inhibits killing of bacteria by bovine PGLYRP-1 (15) and human PGLYRPs, although to a lesser extent than peptidoglycan (M. Wang and R. Dziarski, unpublished results). LTA and LPS bind to PGLYRPs (in addition to peptidoglycan) (10, 12, 15), but the location of LPS- and LTA-binding sites in PGLYRPs is unknown. These sites may be outside the peptidoglycan-binding groove, as suggested by the recently shown binding of ligands outside the peptidoglycan-binding groove in Drosophila PGRP-LE and PGRP-LC (62, 63). In addition, PGRPs have a hydrophobic domain on the opposite side of the molecule from the peptidoglycan-binding groove, which was previously hypothesized to interact with signal transduction molecules in insects (56). In mammalian PGLYRPs, however, this hydrophobic domain may play a role in interaction of PGLYRPs with bacteria. Analogous situation is found in bacteriophage amidases, which lyse peptidoglycan and allow bacteriophages to be released from bacteria. One portion of a bacteriophage amidase is the binding domain specific for a cell wall component often different from peptidoglycan (e.g., choline in pneumococcal cell wall teichoic acid), whereas another portion contains the peptidoglycan-lytic amidase domain (64, 65).

Moreover, binding of PGRPs to bacteria may involve multiple interactions because both human and insect PGRPs use a dual recognition strategy of binding to both MurNAc-pentapeptide and to the peptide cross-bridge (60). Such binding requires interaction of at least two PGRP domains with peptidoglycan. This dual recognition could be accomplished by PGRPs with two PGRP domains and/or by dimeric PGRPs. PGLYRP-1 has one PGRP domain, but it forms disulfide-linked dimers (10), whereas each molecule of PGLYRP-3 and PGLYRP-4 contains two PGRP domains, which are not identical. Moreover, PGLYRP-3 and PGLYRP-4 form homo- or heterodimers (10). Thus, PGLYRP-1 dimers have two identical PGRP domains, whereas PGLYRP-3 and PGLYRP-4 homodimers have two pairs of identical PGRP domains, and PGLYRP-3:4 heterodimers have four different PGRP domains. Therefore, two different PGRP domains and formation of homo- and heterodimers afford PGLYRPs with considerable diversity of specificities and allow highly avid binding to bacteria through interaction with multiple bacterial components. This avid binding may be responsible for the irreversible bactericidal effect of mammalian PGLYRPs.

In conclusion, our results demonstrate that human PGLYRPs are a novel class of innate immunity pattern recognition and effector molecules with Zn2+-dependent broad spectrum bactericidal activity against both Gram-positive and Gram-negative bacteria. At low subbactericidal concentrations, PGLYRPs kill bacteria synergistically with antibacterial peptides, such as PLG, and defensins.

Disclosures
The authors have no financial conflict of interest.

References