Recombinant Human Peptidoglycan Recognition Proteins Reveal Antichlamydial Activity

Pavel Bobrovsky,^a Valentin Manuvera,^{a,c} Nadezhda Polina,^a Oleg Podgorny,^{a,b,c} Kirill Prusakov,^{a,c} Vadim Govorun,^{a,c} Vassili Lazarev^{a,c}

Federal Research and Clinical Center of Physical-Chemical Medicine, Moscow, Russia^a; Koltzov Institute of Developmental Biology of the Russian Academy of Sciences, Moscow, Russia^b; Moscow Institute of Physics and Technology (State University), Dolgoprudny, Moscow Region, Russia^c

Peptidoglycan recognition proteins (PGLYRPs) are innate immune components that recognize the peptidoglycan and lipopolysaccharides of bacteria and exhibit antibacterial activity. Recently, the obligate intracellular parasite *Chlamydia trachomatis* was shown to have peptidoglycan. However, the antichlamydial activity of PGLYRPs has not yet been demonstrated. The aim of our study was to test whether PGLYRPs exhibit antibacterial activity against *C. trachomatis*. Thus, we cloned the regions containing the human *Pglyrp1*, *Pglyrp2*, *Pglyrp3*, and *Pglyrp4* genes for subsequent expression in human cell lines. We obtained stable HeLa cell lines that secrete recombinant human PGLYRPs into culture medium. We also generated purified recombinant PGLYRP1, -2, and -4 and confirmed their activities against Gram-positive (*Bacillus subtilis*) and Gram-negative (*Escherichia coli*) bacteria. Furthermore, we examined the activities of recombinant PGLYRPs against *C. trachomatis* and determined their MICs. We also observed a decrease in the infectious ability of chlamydial elementary bodies in the next generation after a single exposure to PGLYRPs. Finally, we demonstrated that PGLYRPs attach to *C. trachomatis* elementary bodies and activate the expression of the chlamydial two-component stress response system. Thus, PGLYRPs inhibit the development of chlamydial infection.

Peptidoglycan recognition proteins (PGLYRPs) are members of the innate immune system. Initially, they were shown to bind bacterial peptidoglycan and activate the prophenoloxidase pathway in insects (1). Later, PGLYRPs were also found in mammals and other taxonomic groups (2, 3). Mammals have four PGLYRPs, PGLYRP1 to -4, that have been identified as bactericidal proteins (4, 5). PGLYRPs have been shown to recognize not only bacterial peptidoglycan but also lipopolysaccharides in the outer membrane of Gram-negative bacteria (6). In 2010, a novel bacterial killing mechanism that involves PGLYRPs was suggested. PGLYRPs bind to bacterial cell wall peptidoglycan or outer membrane lipopolysaccharides and hyperactivate a stress defense response in bacteria that leads to bacterial suicide (7). PGLYRPs were shown to bind peptidoglycan and lipopolysaccharides in *Bacillus subtilis* and *Escherichia coli*, respectively, and activate the bacterial two-component systems (TCSs) CsxS-CsxR in *B. subtilis* and CpxA-CpxR in *E. coli*, resulting in bacterial death (7). In these TCSs, CsxS and CpxA include a histidine kinase (HK) domain and CsxR and CpxR act as response regulators. In early studies, Lu et al. (8) demonstrated the bactericidal activity of PGLYRPs against different Gram-positive and Gram-negative bacteria *in vitro* and *in vivo*. PGLYRPs exhibit both recognition and effector activities against different bacteria (3, 5, 7–9). However, their activity against intracellular pathogenic parasites such as chlamydiae has not yet been demonstrated.

Chlamydiae alternate between two forms: the infectious, metabolically inactive elementary body (EB) and the noninfectious, metabolically active reticular body (RB) (10). Both the RB and EB forms have lipopolysaccharides (11). Moreover, Liechti et al. (12) reported that *C. trachomatis* has peptidoglycan. It was detected during the transition from the EB form to the RB form (12). These pathogenic bacteria may be considered potential targets for PGLYRPs. Moreover, PGLYRPs have been detected in tissues that have been shown to be attacked by *C. trachomatis* (13, 14). In 2003, Koo and Stephens (15) characterized a pair of genes in *C.*

trachomatis that encode proteins with amino acid sequences similar to those of the bacterial TCS. The pair includes a component with an HK domain (CtcB) and a response regulator protein (CtcC). These proteins were found in EBs of *C. trachomatis*. Thus, the goal of this study was to test whether PGLYRPs exhibit antibacterial activity against the intracellular pathogenic bacterium *C. trachomatis*. Thus, we generated purified human recombinant PGLYRPs, tested their activities against Gram-positive and Gram-negative bacteria, determined their MICs for *C. trachomatis*, and examined the expression of TCS-encoding genes after treating chlamydial EBs with PGLYRPs.

MATERIALS AND METHODS

Bacterial strains and cell lines. HeLa cells (ATCC CCL-2) were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in the presence of 5% CO₂. Expi293F cells (Life Technologies) were cultivated according to the manufacturer's recommendations. *E. coli* Top10 (Invitrogen), *Staphylococcus epidermidis* ATCC 12228, and *Bacillus subtilis* 168 HT were kindly provided by A. Prozorov (Vavilov Institute of General Genetics, Russian Academy of Sciences) and grown in Luria-Bertani (LB) broth at 37°C. The EBs of *C. trachomatis* D/UW-3/Cx (ATCC VR-885) were iso-

Received 21 December 2015 Returned for modification 14 January 2016

Accepted 3 May 2016

Accepted manuscript posted online 9 May 2016

Citation Bobrovsky P, Manuvera V, Polina N, Podgorny O, Prusakov K, Govorun V, Lazarev V. 2016. Recombinant human peptidoglycan recognition proteins reveal antichlamydial activity. Infect Immun 84:2124–2130. doi:10.1128/IAI.01495-15.

Editor: C. R. Roy, Yale University School of Medicine

Address correspondence to Pavel Bobrovsky, pbobrovskiy@gmail.com.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/IAI.01495-15>.

Copyright © 2016, American Society for Microbiology. All Rights Reserved.

lated by urografin gradient ultracentrifugation as described previously (16). For *C. trachomatis* growth in HeLa cells, DMEM with 0.5% glucose, 10% FBS, and 25 µg/ml gentamicin was used. Before *C. trachomatis* inoculation, the HeLa cells were seeded into 24-well plates (Corning) at approximately 10^5 /well and cultured for 24 h to achieve monolayer confluence. The cell monolayer was then infected with a *C. trachomatis* inoculum at multiplicities of infection (MOIs) ranging from 0.9 to 1. The cells were centrifuged for 1 h at $900 \times g$.

For protein production, the HeLa-PGLYRP cells were incubated overnight, washed three times with DMEM, and then cultured in DMEM lacking FBS and containing 10 µg/ml gentamicin. The cells were cultivated for an additional 72 h at 37°C in the presence of 5% CO₂.

PCR. PCR was performed in a DNA Engine Tetrad 2 Thermal Cycler (Bio-Rad) in a reaction volume of 25 µl with 2 to 3 mM MgCl₂, 0.2 mM each deoxynucleoside triphosphate (dNTP), 67 mM Tris-HCl (pH 8.3), 16.7 mM (NH₄)₂SO₄, 0.5 U of *Taq* DNA polymerase (Thermo Scientific), 1 to 10 ng of DNA, and 5 pmol of each primer. The thermal cycling conditions (°C/s) were 95/120 for 1 cycle and 95/10, 55 to 62/10, and 72/20 to 120 for 25 cycles, with a final elongation of 72/120.

Reverse transcription. Total RNA was isolated with TRIzol (Invitrogen) according to the manufacturer's instructions. Because of the tissue specificity of PGLYRPs (14), white blood cells were used to obtain *Pglyrp1* mRNA. The HepG2 cell line was used to obtain *Pglyrp2* mRNA, and biopsy material from a human esophagus was used to obtain *Pglyrp3* and *Pglyrp4* mRNA. DNA contamination was removed by DNase digestion with DNase I (Thermo Scientific). cDNA was then synthesized by reverse transcription with Moloney murine leukemia virus reverse transcriptase (Promega), according to the manufacturer's protocol, and the polyT primer (see Table S1 in the supplemental material).

Cloning of the *Pglyrp* coding regions. cDNA was amplified with specific oligonucleotides (for the sequences, see Table S1 in the supplemental material). To obtain expression vectors, the PGLYRP genes were cloned into the pEGFP-N1 (Clontech) and pcDNA3.4 TOPO (Life Technologies) vectors. Thus, we constructed pcDNA-IGG-PGLYRP plasmids encoding *Pglyrp* fused with the rabbit IgG light chain, a tobacco etch virus (TEV) protease cleavage site, and a C-terminal 6His tag. Plasmid DNA was isolated and verified by sequencing with the BigDye Terminator Cycle Sequencing kit (v.3.1) on an ABI PRISM 3730xl Gene Analyzer (Applied Biosystems) with oligonucleotides pN1-pN2 for all pEGFP-N1-PGLYRP vectors and Cmv_F-pcDNA_R for all pcDNA3.4-PGLYRP vectors. For the cloning schemes, see Fig. S1 to S3 in the supplemental material.

Transfection of HeLa and Expi293F cells. Expi293F cells were cultivated and transfected with pcDNA-IGG-PGLYRP vectors according to the manufacturer's instructions. HeLa cells were transfected with pEGFP-N1-PGLYRP vectors and the TransPass COS/293 Transfection Reagent (NEB) according to the manufacturer's instructions, and stably transfected clones were selected with 500 µg/ml G418 (Life Technologies).

Generation of PGLYRP1, -2, and -4 from fusion protein light chain Igg-PGLYRP. Expi293F cells transfected with pcDNA-IGG-PGLYRP plasmids were cultivated in 100-ml flasks with a final volume of 30 ml of Expi293 culture medium. At 120 h after transfection, the cell culture was centrifuged at $130 \times g$ for 10 min. The culture medium was diluted in 8× chromatographic buffer (final concentrations of 500 mM NaCl, 20 mM NaH₂PO₄, and 10 mM imidazole [pH 7.4]) and subsequently centrifuged ($12,000 \times g$ for 15 min). The final solution was applied to a column containing 1 ml of Ni Sepharose High Performance (GE Healthcare) equilibrated with chromatographic buffer. The column was washed with 200 ml of washing buffer (500 mM NaCl, 20 mM NaH₂PO₄, 40 mM imidazole, pH 7.4), and then the bound fraction was eluted with eluting buffer (500 mM NaCl, 20 mM NaH₂PO₄, 500 mM imidazole, pH 7.4). The flow rate was 1.5 ml/min. The proteins were purified with the AKTA fast protein liquid chromatography system (GE Healthcare). The solution of the fusion protein was dialyzed against phosphate-buffered saline (PBS, pH 7.4) at 4°C overnight. The protein concentration was measured with the Bradford protein assay. Next, 1% (wt/wt) TEV protease (Sigma) and

0.5% (vol/vol) 2-mercaptoethanol were added and the mixture was subsequently incubated at room temperature overnight. After incubation, the solution was centrifuged at $12,000 \times g$ for 15 min. The supernatant was diluted with chromatographic buffer to a final volume of 50 ml and applied to a Ni Sepharose column for purification as described previously. The presence of PGLYRP in the collected fractions was determined by Laemmli SDS-PAGE.

As controls for subsequent experiments with purified PGLYRPs, we used blank control samples and elution buffer. To prepare the blank control sample, we applied culture medium from Expi293F cells transfected with the pcDNA_IGG vector to the Ni Sepharose column. We then dialyzed the eluate against PBS, pH 7.4, at 4°C overnight. We added TEV protease and 2-mercaptoethanol to the solution at the same concentrations used for PGLYRP proteolysis. The solution was incubated overnight, diluted 10-fold, and then applied to the Ni Sepharose column. The eluate served as the blank control sample.

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) analysis. The protein bands after one-dimensional SDS-PAGE were exposed to trypsin in-gel hydrolysis. Gel pieces (2 to 3 mm³) were cut and washed twice with 100 µl of 0.1 M ammonium bicarbonate and 40% acetonitrile for 30 min at 37°C, dehydrated with 100 µl of acetonitrile, and air dried. Gel pieces were then treated with 4 µl of a 12.5-mg/ml solution of modified trypsin (Promega) in 40 mM ammonium bicarbonate and 10% acetonitrile for 16 h at 37°C. The peptides were extracted with 8 µl of an aqueous solution of 0.5% trifluoroacetic acid for 20 min. Sample aliquots (2 µl) were mixed on a steel target with 0.3 µl of a 2,5-dihydroxybenzoic acid (Bruker Daltonics) solution (75 mg/ml in 30% acetonitrile–0.5% trifluoroacetic acid). Mass spectra were recorded on an Ultraflex II MALDI-TOF-TOF mass spectrometer (Bruker Daltonics) equipped with an Nd laser. Molecular ions were measured in the reflector mode; the accuracy of the mass peak measurement was 0.005%. The fragment ion spectra were generated by laser-induced dissociation accelerated by low-energy collision-induced dissociation with helium as the collision gas. The accuracy of the fragment ion mass peak measurement was 5 Da. The tandem mass spectrometry (MS/MS) fragments were identified with BioTools software (Bruker Daltonics) and Mascot MS/MS ion search. Protein identification was performed by a peptide fingerprint search with Mascot software (Matrix Science Inc.). One missed cleavage, Cys-propionamide, and Met oxidation were permitted. Protein scores of >49 were considered significant ($P < 0.05$).

Determination of MICs for *E. coli* and *B. subtilis*. MICs were determined as the lowest concentrations of PGLYRPs that inhibit the visible growth of bacteria. The broth dilution method was used according to reference 17. Briefly, 150 µl of LB medium containing *E. coli* or *B. subtilis* at 5×10^5 CFU/ml was added to the wells of a 96-well plate. Serial 2-fold dilutions of PGLYRPs in Hanks' buffered salt solution (HBSS) (Thermo Scientific) were prepared and added to the wells for a final volume of 200 µl. The plates were incubated at 37°C for 18 h. Growth patterns in plates were analyzed, and MICs were determined as previously described (17).

Determination of the MICs for *C. trachomatis*. Measurements of the MICs for *C. trachomatis* were performed as follows. HeLa cells were seeded into the wells of a 24-well plate with coverslips and cultivated until 90% confluence was achieved. We prepared serial 2-fold dilutions of PGLYRPs. Proteins were diluted in HBSS to a final volume of 199 µl. One microliter of *C. trachomatis* inoculum (10^5 EBs/µl) was added to each PGLYRP sample, which was then incubated while shaking at 37°C for 2 h. Culture medium was aspirated from the wells seeded with HeLa cells, and 200 µl of PGLYRP samples with chlamydial EBs or control samples were added to the cells. The plates were centrifuged ($900 \times g$, 1 h), and fresh DMEM was added to a final volume of 1 ml. We added HBSS and a blank control sample in HBSS to control wells. Plates were cultivated at 37°C in the presence of 5% CO₂ for an additional 47 h. After incubation, wells were fixed with 4% paraformaldehyde for 20 min and permeabilized with 1% Triton X-100 for 30 min. The cells were stained by direct immunofluorescence with chicken anti-chlamydial lipooligosaccharide antibodies la-

beled with fluorescein isothiocyanate (FITC; Galart Diagnosticum) for 1 h. The antibody solution also contained ethidium bromide. Stained coverslips were placed onto glass slides with mounting medium. Images of 10 randomly selected fields for each coverslip were captured by a Nikon Eclipse E800 confocal microscope (Nikon, Japan) with excitation at 488 nm (FITC channel) and 594 nm (ethidium bromide channel), a 10× Plan Fluor objective, and a 40× Plan Fluor objective. The numbers of labeled cells in both channels were quantified with ImageJ software (version 1.48; RSB [<http://imagej.nih.gov/>]). The infection rate was determined as the ratio of the number of cells with antibody-labeled inclusions (FITC channel) to the total number of cells (ethidium bromide channel). MICs were determined as the lowest concentrations of PGLYRPs at which the infection rate was reduced to >90% of that in the control wells (18).

Production of infectious progeny. To test the action of PGLYRPs on infectious progeny production, we seeded HeLa cells into two 24-well plates and cultivated them until 90% confluence was achieved. In each plate, we added an aliquot of PGLYRPs (100 ng/ml), as well as a blank control sample of equal volume to the control wells. The cells in both plates were infected with *C. trachomatis* at an MOI of 1, as described above, and cultured at 37°C for an additional 71 h. One of the plates was then fixed and used to count the inclusion-forming units (IFUs). Titers (in IFUs per milliliter) were calculated as described previously (16). In another plate, the culture medium was removed from the wells. Cells were resuspended in 200 μ l of HBSS by pipetting, transferred to microcentrifuge tubes, and lysed by freezing/thawing. To prepare inocula with equivalent MOIs for the next round of infection, we diluted lysates according to titers that had been calculated by using the first plate. Diluted lysates were used to infect fresh HeLa cells. The cells were then fixed at 48 h postinoculation. IFUs were counted, and titers were calculated as described above.

PGLYRP attachment to *C. trachomatis*. To prove that PGLYRPs attach to *C. trachomatis*, solutions containing purified PGLYRPs (50 ng in 100 μ l of HBSS) were mixed with 30 μ l of *C. trachomatis* inoculum (10^5 cells/ μ l). We used purified PGLYRPs in HBSS and EBs in HBSS as control samples. Samples were centrifuged at $20,000 \times g$ at 4°C for 30 min. The supernatant was collected in a fresh tube. The sediment was washed with HBSS, centrifuged at $20,000 \times g$ for 10 min at 4°C, and diluted in 100 μ l of HBSS. Equal aliquots of sediment and supernatant were analyzed by Western blotting with mouse anti-6His monoclonal antibody to detect the attachment of *C. trachomatis* and PGLYRPs.

Western blotting. PGLYRPs diluted in Laemmli sample buffer were heated to 95°C for 10 min. The probes were separated by 12% SDS-PAGE. After separation, the proteins were transferred (semidry transfer, 1 mA/cm² for 1 h) to a polyvinylidene difluoride membrane (GE Healthcare) in Tris-glycine transfer buffer (48 mM Tris, 39 mM glycine, 0.04% SDS, 20% methanol). The membrane was blocked with 3% nonfat dry milk in PBS and treated with mouse anti-6His monoclonal antibody at a 1:10,000 dilution (Invitrogen) at 4°C overnight. The membrane was washed three times with 0.1% Tween 20 in PBS, incubated with a horseradish peroxidase-linked sheep anti-mouse IgG antibody at a 1:25,000 dilution (GE Healthcare) for 1 h, and then washed three times. The membrane was treated with ECL Plus Western blotting detection reagents (GE Healthcare) according to the manufacturer's instructions. The signals were detected with the ChemiDoc XRS+ System (Bio-Rad).

Analysis of chlamydial TCS gene expression activation. HeLa cells were seeded into the wells of a 24-well plate and cultivated until 90% confluence was achieved. PGLYRPs (100 ng/ml) were diluted in HBSS to a final volume of 199 μ l. One microliter of *C. trachomatis* inoculum (10^5 EBs/ μ l) was added to each PGLYRP sample. We added HBSS and blank control samples in HBSS to control wells. The culture medium was aspirated from the wells seeded with HeLa cells, and 200 μ l of PGLYRP samples with chlamydial EBs or control samples were added to the cells. The cells were infected with *C. trachomatis* EBs at an MOI of 1. The cells were centrifuged ($900 \times g$, 1 h). Total RNA was isolated at different postinoculation time points ranging from 0 to 72 h. cDNA was obtained with specific primers (CtcC_R, CtcB_R, EuoR, and OmcB_R). Real-time PCR

FIG 1 Western blot analysis of recombinant human PGLYRPs from HeLa cells transfected with pEGFP-N1-PGLYRP vectors. Lanes: a, PGLYRP1; b, PGLYRP2; c, PGLYRP3; d, PGLYRP4; M, molecular mass markers (corresponding values [in kilodaltons] are shown on the left).

was performed with the CtcB_F-CtcB_R, CtcC_F-CtcC_R, EuoF-EuoR, and OmcB_F-OmcB-R primers (see Table S1 in the supplemental material). Real-time PCR was performed with the CFX96 Touch Real-Time PCR detection system (Bio-Rad) with $0.1 \times$ SYBR green I (Invitrogen), $1 \times$ Taq buffer with $(\text{NH}_4)_2\text{SO}_4$ (Thermo Scientific), 0.2 mM each dNTP, 0.5 U of Taq DNA polymerase (Thermo Scientific), 10 ng of cDNA, and each primer at 5 pM. The data were normalized to the early upstream open reading frame protein (*euo*) and large cysteine-rich periplasmic protein (*omcB*) reference genes and by calculation of the geometric mean of three repeats as described in reference 19. Fold changes were determined as ratios of gene expression levels at different time points to those at the 0-h time point.

Statistics. Data analysis was performed via parametric statistical analysis (Student's *t* test and multiple-comparison test [two-way analysis of variance]) with Statistica software (version 8.0; StatSoft Inc.). Data are presented as means $\pm$ standard deviations. Differences among means were considered significant at a *P* value of <0.05.

RESULTS

Transient transfection and protein identification. To obtain recombinant human PGLYRPs, we generated stable HeLa cell lines that secrete PGLYRPs into culture medium. These lines were obtained by transfection of HeLa cells with pEGFP-N1-PGLYRP vectors (see Fig. S1 in the supplemental material). Transfected cells were selected with G418, and the expression of PGLYRP genes was confirmed by reverse transcription-PCR. We detected recombinant proteins in the culture supernatant by Western blotting (Fig. 1). However, we could not obtain purified proteins because of low levels of accumulation. Next, we transiently transfected Expi293 cells with pcDNA-PGLYRP vectors (see Fig. S2 in the supplemental material). The production of PGLYRPs in culture medium was confirmed by Western blotting (not shown). In this case, we also could not obtain purified proteins because of a low yield. Thus, we constructed new vectors, pcDNA-IGG-PGLYRPs (see Fig. S3 in the supplemental material), encoding rabbit IgG light chain fused to PGLYRPs, interspaced with the TEV protease cleavage site. The transient transfection of Expi293 cells with pcDNA-IGG-PGLYRP vectors caused enhanced secretion of recombinant proteins into the culture medium, which subsequently allowed us to purify recombinant human PGLYRP1, -2, and -4 by chromatography. The level of accumulated recombinant PGLYRP3 was insufficient for subsequent purification.

Fused proteins underwent proteolysis with TEV protease (20)

FIG 2 Coomassie G-250-stained Laemmli SDS-PAGE analysis of recombinant PGLYRPs isolated from culture medium supernatants of Expi293F cells transfected with pcDNA-IGG-PGLYRP vectors. Lanes: a to c, purified PGLYRP1, -2, and -4, respectively; m, molecular mass markers (corresponding values [in kilodaltons] are shown on the right).

in the presence of 0.5% 2-mercaptoethanol. The optimal substrate-to-protease ratio was 100:1 (wt/wt). In this case, almost complete cleavage occurred after overnight incubation. Because of the high 2-mercaptoethanol concentration, the samples were diluted 10-fold to avoid Ni^{2+} reduction during subsequent chromatographic purification. Purified proteins were analyzed by SDS-PAGE (Fig. 2) and identified by MALDI-TOF analysis (see Fig. S4 in the supplemental material). The final yield of pure PGLYRP1, -2, and -4 was approximately 50 mg/liter of the initial cell culture.

MIC determination. To test the antibacterial activities of generated recombinant proteins, we measured their MICs for *E. coli* and *B. subtilis* (Table 1). The results obtained allowed us to confirm the presence of PGLYRP antibacterial activity. Furthermore, we determined the MICs of PGLYRPs for *C. trachomatis* (Table 1).

PGLYRP attachment to *C. trachomatis*. We tested whether PGLYRPs attach to *C. trachomatis*. To this end, we analyzed four samples by Western blotting with mouse anti-6His antibody (Fig. 3). The first sample was purified PGLYRP1 in HBSS (Fig. 3, lane a), the second sample was the supernatant of a centrifuged mixture of PGLYRP1 with chlamydial EBs in HBSS (Fig. 3, lane b), the third sample was sediment of a centrifuged mixture of PGLYRP1 with chlamydial EBs in HBSS (Fig. 3, lane c), and the fourth sample was sediment of centrifuged chlamydial EBs in HBSS (Fig. 3, lane d). We found that PGLYRP1 was detected predominantly in the sediment after the centrifugation of a mixture of PGLYRPs with chlamydial EBs (Fig. 3, lanes a and c, PGLYRP bands). We also observed nonspecific staining of chlamydial proteins (Fig. 3, lanes c and d, U.B. bands). Similar results were obtained with PGLYRP2 and -4. The detection of PGLYRPs in the sediment after the centrifugation of a mixture of PGLYRPs with chlamydial EBs confirmed the attachment of PGLYRPs to chlamydial EBs.

TABLE 1 MICs of PGLYRP1, PGLYRP2, and PGLYRP4 determined for *E. coli*, *B. subtilis*, and *C. trachomatis*

Protein	MIC (ng/ml) for:		
	<i>E. coli</i>	<i>B. subtilis</i>	<i>C. trachomatis</i>
PGLYRP1	12.5	12.5	200
PGLYRP2	25	25	400
PGLYRP4	12.5	12.5	200

FIG 3 Western blot analysis of the attachment of recombinant PGLYRP1 to *C. trachomatis*. Lanes: a, purified PGLYRP1 in HBSS; b, supernatant of a centrifuged mixture of PGLYRP1 with chlamydial EBs in HBSS; c, sediment of a centrifuged mixture of PGLYRP1 with chlamydial EBs in HBSS; d, sediment of centrifuged chlamydial EBs in HBSS. The upper unspecific bands (U.B.) correspond to nonspecific staining of chlamydial proteins. Lane M, molecular mass markers (corresponding values [in kilodaltons] are shown on the right).

Production of infectious progeny. To test whether PGLYRPs inhibit the production of infectious progeny, we added PGLYRPs to HeLa cells and infected them with *C. trachomatis* EBs. At the first round of infection, we found decreased chlamydial titers in all of the wells to which PGLYRPs had been added before inoculation of *C. trachomatis* EBs (Fig. 4A). Because of the difference in the titers between samples, inocula obtained after the first round of infection were diluted to achieve equivalent MOIs. Diluted inocula were used for the second round of infection of fresh HeLa cells. Forty-eight hours postinoculation, we detected increased titers in the control wells compared to the initial titers (Fig. 4B). This observation shows the growth of chlamydia in control wells. In the wells to which PGLYRPs were added before inoculation of *C. trachomatis* EBs, we detected titers lower than the initial titers (Fig. 4B). Thus, our results demonstrate that PGLYRPs inhibit chlamydial growth and decrease infectious progeny production.

Analysis of chlamydial TCS gene expression activation. To test whether PGLYRPs activate the chlamydial TCS, we analyzed a time course of *ctcB* and *ctcC* expression after the infection of HeLa cells with EBs treated with PGLYRPs. We used a blank control sample in HBSS as a control. We isolated total RNA from infected HeLa cells at 0, 1, 24, 48, 60, and 72 h postinoculation. Real-time PCR analysis showed increased levels of the *ctcB* and *ctcC* genes at 1 and 72 h postinoculation in cases where EBs had been treated with PGLYRPs (Fig. 5). Furthermore, we increased the time resolution by using 0-, 15-, 30-, 60-, 90-, 120-, 180-, and 300-min time points postinoculation for analysis. Maximal levels of the *ctcB* and *ctcC* genes were observed at 2 h postinoculation (Fig. 6). In the control samples, we detected no significant differences at the time points indicated (Fig. 5 and 6). Thus, treatment of chlamydial EBs with PGLYRPs activates the expression of genes that participate in the two-component stress response system.

DISCUSSION

In the present study, we obtained soluble recombinant human peptidoglycan recognition proteins in the Expi293F human cell line, with yields of 50 mg/liter of culture medium. Because the stable cell lines and transient transfection with gene constructions encoding PGLYRPs gave insufficient yields for the purification of proteins, we then fused PGLYRPs with IgG light chains. These fused proteins were interspaced with the TEV protease site for

FIG 4 Changes in parental and progeny *C. trachomatis* titers after treatment with purified separated PGLYRPs added to HeLa cells. The parental titers were calculated at 72 h postinfection (A). To normalize the data from parental titers, samples were diluted to achieve equivalent MOIs (horizontal line) to analyze the production of infectious progeny (B). We used a blank control sample as a control.

further cleavage and purification. The recombinant fusion proteins were detected in the culture medium of Expi293F cells transfected with the pcDNA3.4-IGG-PGLYRP. They were then purified from the culture medium and separated from the light chain of IgG by proteolysis (Fig. 2). Thus, we have developed a protocol for the generation of pure PGLYRPs in Expi293F cells.

Previously, recombinant human PGLYRPs produced in *Drosophila* S2 and monkey COS-7 cells were shown to have bactericidal activity against different bacteria (8). We tested the antibacterial activity of PGLYRPs expressed in a homologous expression system. These proteins demonstrated activity against Gram-positive *B. subtilis* and Gram-negative *E. coli*, and the MICs determined in our study (Table 1) prove the activity of PGLYRPs against bacteria. Furthermore, we determined the MICs of PGLYRPs for *C. trachomatis*. In this case, the MICs were found to be approximately 20 times those for *E. coli* and *B. subtilis*. These

larger values may be associated with the specific lifestyle of chlamydiae. To date, few antimicrobial proteins and peptides are known to possess antichlamydial activity. Several studies have shown the antichlamydial effects of host immune effectors such as defensins (21–23), cathelicidin peptide LL-37 (22, 24), and protegrin (23). The pure-PGLYRP MICs obtained in our study may prove the activity of PGLYRPs against *C. trachomatis*.

It is unknown how PGLYRPs kill *C. trachomatis*. Because PGLYRPs have been shown to bind the lipopolysaccharides of different bacteria (4, 7, 25), they may bind the chlamydial lipopolysaccharides that are known to be present in both RBs and EBs (11). The main question was whether PGLYRPs attach to *C. trachomatis*. Using precipitation and Western blot analysis, we demonstrated conclusively that PGLYRPs interact directly with *C. trachomatis* EBs (Fig. 3).

We do not know the exact effect of PGLYRPs on *C. trachoma-*

FIG 5 Analysis of the activation of chlamydial two-component stress response system gene expression after treatment with purified recombinant PGLYRPs at 1, 24, 48, 60, and 72 h postinoculation. The quantitative PCR data were normalized, and the fold change in the mRNA level was determined as $2^{-\Delta\Delta CT}$. Data are expressed as the mRNA fold change $\pm$ the standard error. A blank control sample was used as a control. Differences from the untransfected control were considered significant if the *P* value was <0.05 (*).

FIG 6 Analysis of the activation of chlamydial two-component stress response system-related gene expression after treatment with PGLYRPs at 15, 30, 60, 90, 120, 180, and 300 min postinoculation. The quantitative PCR data were normalized, and the fold change in the mRNA level was determined as $2.1^{-\Delta\Delta CT}$. A blank control sample was used as a control. Data are expressed as fold changes in mRNA levels $\pm$ the standard errors.

tis. We found that a single contact between *C. trachomatis* and PGLYRPs not only decreases the infectious ability of initial EBs but also influences the production of infectious progeny (Fig. 4). It was shown that the membrane of EBs is impermeable to most molecules (large proteins) (26, 27). Thus, it is unlikely that PGLYRPs penetrate EBs. In addition, it is unlikely that PGLYRPs act from within. Likely, PGLYRPs act outside eukaryotic cells, likely influencing both the differentiation of the EB form into the RB form and the subsequent differentiation of the RB form back into the EB form.

It was previously shown that the bacterial stress defense response to PGLYRPs is carried out by the two-component stress response system (7). TCSs detect translocated extracytoplasmic misfolded or aggregated proteins (28, 29). Binding of PGLYRPs to peptidoglycan or lipopolysaccharides has been shown to provoke activation of the two-component stress response system (30). Some bacterial TCSs have an autoactivation mechanism (29) that involves activation of TCS gene expression after activation of TCS proteins. This may explain the overactivation of the stress response. *C. trachomatis* also has a two-component stress response system (CtcB-CtcC) (15). We showed that contact of PGLYRPs with *C. trachomatis* EBs activates the expression of genes encoding the proteins of the two-component stress response system. This may explain the inhibition of chlamydial infection. Initially, we detected two peaks corresponding to high *ctcC* and *ctcB* mRNA levels (1 h and 72 h). The 1-h peak corresponds to TCS gene expression activation after contact between PGLYRPs and EBs. We also detected a small increase in the mRNA level at the 60-h time point that may be associated with the initiation of EB release from HeLa cells. We then detected a second 72-h peak corresponding to the maximum release of EBs. This peak may be explained by the interaction of released EBs with PGLYRPs in culture medium during the second round of infection (31). Furthermore, we increased the time resolution and found that maximal *ctcC* and *ctcB* mRNA levels were achieved at 2 h after contact between EBs and PGLYRPs (Fig. 5 and 6).

In summary, our results demonstrate the antichlamydial activity of recombinant human PGLYRPs, which is associated with the activation of genes involved in the chlamydial two-component stress response system.

ACKNOWLEDGMENTS

We thank Sergey Kovalchuk (Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences, Moscow, Russia), Olga Pobeguts, and Dariya Matushkina (Federal Research and Clinical Centre of Physical-Chemical Medicine, Moscow, Russia) for the MALDI-TOF analyses.

This research received no specific grants from any funding agency in the public, commercial, or not-for-profit sector.

REFERENCES

- Yoshida H, Kinoshita K, Ashida M. 1996. Purification of a peptidoglycan recognition protein from hemolymph of the silkworm, *Bombyx mori*. *J Biol Chem* 271:13854–13860. <http://dx.doi.org/10.1074/jbc.271.23.13854>.
- Kang D, Liu G, Lundstrom A, Gelius E, Steiner H. 1998. A peptidoglycan recognition protein in innate immunity conserved from insects to humans. *Proc Natl Acad Sci U S A* 95:10078–10082. <http://dx.doi.org/10.1073/pnas.95.17.10078>.
- Liu C, Xu Z, Gupta D, Dziarski R. 2001. Peptidoglycan recognition proteins: a novel family of four human innate immunity pattern recognition molecules. *J Biol Chem* 276:34686–34694. <http://dx.doi.org/10.1074/jbc.M105566200>.
- Royet J, Dziarski R. 2007. Peptidoglycan recognition proteins: pleiotropic sensors and effectors of antimicrobial defences. *Nat Rev Microbiol* 5:264–277. <http://dx.doi.org/10.1038/nrmicro1620>.
- Wang ZM, Li X, Cocklin RR, Wang M, Fukase K, Inamura S, Kusumoto S, Gupta D, Dziarski R. 2003. Human peptidoglycan recognition protein-L is an *N*-acetylmuramoyl-L-alanine amidase. *J Biol Chem* 278:49044–49052. <http://dx.doi.org/10.1074/jbc.M307758200>.
- Tydel CC, Yuan J, Tran P, Selsted ME. 2006. Bovine peptidoglycan recognition protein-S: antimicrobial activity, localization, secretion, and binding properties. *J Immunol* 176:1154–1162. <http://dx.doi.org/10.4049/jimmunol.176.2.1154>.
- Dziarski R, Kashyap DR, Gupta D. 2012. Mammalian peptidoglycan recognition proteins kill bacteria by activating two-component systems and modulate microbiome and inflammation. *Microb Drug Resist* 18:280–285. <http://dx.doi.org/10.1089/mdr.2012.0002>.
- Lu X, Wang M, Qi J, Wang H, Li X, Gupta D, Dziarski R. 2006. Peptidoglycan recognition proteins are a new class of human bactericidal proteins. *J Biol Chem* 281:5895–5907.
- Royet J, Gupta D, Dziarski R. 2011. Peptidoglycan recognition proteins: modulators of the microbiome and inflammation. *Nat Rev Immunol* 11:837–851. <http://dx.doi.org/10.1038/nri3089>.
- Moulder JW. 1991. Interaction of chlamydiae and host cells in vitro. *Microbiol Rev* 55:143–190.
- Hearn SA, McNabb GL. 1991. Immunoelectron microscopic localization of chlamydial lipopolysaccharide (LPS) in McCoy cells inoculated with

- Chlamydia trachomatis*. J Histochem Cytochem 39:1067–1075. <http://dx.doi.org/10.1177/39.8.1649854>.
12. Liechti GW, Kuru E, Hall E, Kalinda A, Brun YV, VanNieuwenhze M, Maurelli AT. 2014. A new metabolic cell-wall labelling method reveals peptidoglycan in *Chlamydia trachomatis*. Nature 506:507–510. <http://dx.doi.org/10.1038/nature12892>.
 13. Fields S, Song B, Rasoul B, Fong J, Works MG, Shew K, Yiu Y, Mirsalis J, D'Andrea A. 2014. New candidate biomarkers in the female genital tract to evaluate microbicide toxicity. PLoS One 9:e110980. <http://dx.doi.org/10.1371/journal.pone.0110980>.
 14. Dziarski R, Gupta D. 2006. The peptidoglycan recognition proteins (PGRPs). Genome Biol 7:232. <http://dx.doi.org/10.1186/gb-2006-7-8-232>.
 15. Koo IC, Stephens RS. 2003. A developmentally regulated two-component signal transduction system in *Chlamydia*. J Biol Chem 278:17314–17319. <http://dx.doi.org/10.1074/jbc.M212170200>.
 16. Scidmore MA. 2005. Cultivation and laboratory maintenance of *Chlamydia trachomatis*. Curr Protoc Microbiol Chapter 11:Unit 11A.1. <http://dx.doi.org/10.1002/9780471729259.mc11a01s00>.
 17. Wiegand I, Hilpert K, Hancock RE. 2008. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc 3:163–175. <http://dx.doi.org/10.1038/nprot.2007.521>.
 18. Malay S, Roblin PM, Reznik T, Kutlin A, Hammerschlag MR. 2002. In vitro activities of BMS-284756 against *Chlamydia trachomatis* and recent clinical isolates of *Chlamydia pneumoniae*. Antimicrob Agents Chemother 46:517–518. <http://dx.doi.org/10.1128/AAC.46.2.517-518.2002>.
 19. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:RESEARCH0034.
 20. van den Berg S, Lofdahl PA, Hard T, Berglund H. 2006. Improved solubility of TEV protease by directed evolution. J Biotechnol 121:291–298. <http://dx.doi.org/10.1016/j.jbiotec.2005.08.006>.
 21. Donati M, Di Leo K, Benincasa M, Cavrini F, Accardo S, Moroni A, Gennaro R, Cevenini R. 2005. Activity of cathelicidin peptides against *Chlamydia* spp. Antimicrob Agents Chemother 49:1201–1202. <http://dx.doi.org/10.1128/AAC.49.3.1201-1202.2005>.
 22. Hou S, Dong X, Yang Z, Li Z, Liu Q, Zhong G. 2015. Chlamydial plasmid-encoded virulence factor Pgp3 neutralizes the antichlamydial activity of human cathelicidin LL-37. Infect Immun 83:4701–4709. <http://dx.doi.org/10.1128/IAI.00746-15>.
 23. Tang L, Chen J, Zhou Z, Yu P, Yang Z, Zhong G. 2015. Chlamydia-secreted protease CPAF degrades host antimicrobial peptides. Microbes Infect 17:402–408. <http://dx.doi.org/10.1016/j.micinf.2015.02.005>.
 24. Donati M, Di Francesco A, Gennaro R, Benincasa M, Magnino S, Pignatelli S, Shurdhi A, Moroni A, Mazzoni C, Meriardi G, Baldelli R, Cevenini R. 2007. Sensitivity of *Chlamydia suis* to cathelicidin peptides. Vet Microbiol 123:269–273. <http://dx.doi.org/10.1016/j.vetmic.2007.02.011>.
 25. Dziarski R, Gupta D. 2010. Review: mammalian peptidoglycan recognition proteins (PGRPs) in innate immunity. Innate Immun 16:168–174. <http://dx.doi.org/10.1177/1753425910366059>.
 26. Bavoil P, Ohlin A, Schachter J. 1984. Role of disulfide bonding in outer membrane structure and permeability in *Chlamydia trachomatis*. Infect Immun 44:479–485.
 27. Heinzen RA, Hackstadt T. 1997. The *Chlamydia trachomatis* parasitophorous vacuolar membrane is not passively permeable to low-molecular-weight compounds. Infect Immun 65:1088–1094.
 28. Darmon E, Noone D, Masson A, Bron S, Kuipers OP, Devine KM, van Dijk JM. 2002. A novel class of heat and secretion stress-responsive genes is controlled by the autoregulated CsxRS two-component system of *Bacillus subtilis*. J Bacteriol 184:5661–5671. <http://dx.doi.org/10.1128/JB.184.20.5661-5671.2002>.
 29. De Wulf P, Kwon O, Lin EC. 1999. The CpxRA signal transduction system of *Escherichia coli*: growth-related autoactivation and control of unanticipated target operons. J Bacteriol 181:6772–6778.
 30. Kashyap DR, Wang M, Liu LH, Boons GJ, Gupta D, Dziarski R. 2011. Peptidoglycan recognition proteins kill bacteria by activating protein-sensing two-component systems. Nat Med 17:676–683. <http://dx.doi.org/10.1038/nm.2357>.
 31. Lyons JM, Ito JI, Jr, Pena AS, Morre SA. 2005. Differences in growth characteristics and elementary body associated cytotoxicity between *Chlamydia trachomatis* oculogenital serovars D and H and *Chlamydia muridarum*. J Clin Pathol 58:397–401. <http://dx.doi.org/10.1136/jcp.2004.021543>.