Mammalian Peptidoglycan Recognition Protein Binds Peptidoglycan with High Affinity, Is Expressed in Neutrophils, and Inhibits Bacterial Growth*

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Peptidoglycan recognition protein (PGRP) is conserved from insects to mammals. In insects, PGRP recognizes bacterial cell wall peptidoglycan (PGN) and activates prophenoloxidase cascade, a part of the insect antimicrobial defense system. Because mammals do not have the prophenoloxidase cascade, its function in mammals is unknown. However, it was suggested that an identical protein (Tag7) was a tumor necrosis factor-like cytokine. Therefore, the aim of this study was to identify the function of PGRP in mammals. Mouse PGRP bound to PGN with fast kinetics and nanomolar affinity ($K_d = 13$ nM). The binding was specific for polymeric PGN or Gram-positive bacteria with unmodified PGN, and PGRP did not bind to other cell wall components or Gram-negative bacteria. PGRP mRNA and protein were expressed in neutrophils and bone marrow cells, but not in spleen cells, mononuclear cells, T or B lymphocytes, NK cells, thymocytes, monocytes, and macrophages. PGRP was not a PGN-lytic or a bacteriolytic enzyme, but it inhibited the growth of Gram-positive but not Gram-negative bacteria. PGRP inhibited phagocytosis of Gram-positive bacteria by macrophages, induction of oxidative burst by Gram-positive bacteria in neutrophils, and induction of cytokine production by PGN in macrophages. PGRP had no tumor necrosis factor-like cytotoxicity for mammalian cells, and it was not chemoattractant on its own or in combination with PGN. Therefore, mammalian PGRP binds to PGN and Gram-positive bacteria with nanomolar affinity, is expressed in neutrophils, and inhibits growth of bacteria.

Innate immunity is the first line of defense directed against components common to microorganisms (1, 2). One of the antimicrobial mechanisms of innate immunity in insects is the prophenoloxidase cascade, which is present in hemolymph and cuticle (3). The prophenoloxidase cascade can be initiated by the binding of a 19-kDa protein, peptidoglycan-recognition protein (PGRP), to a bacterial cell wall component, peptidoglycan (PGN) (4).

Recently, insect PGRP as well as mouse and human PGRP homologs were cloned and shown to bind Gram-positive bacteria and PGN (5), thus demonstrating that this protein is highly conserved from insects to mammals and providing additional evidence for the highly conserved nature of innate immunity from insects to mammals. Very high expression of mammalian PGRP mRNA in the bone marrow and lower expression in other lymphoid tissues (5) suggested a role for mammalian PGRP in immunity. However, the exact cell types expressing PGRP and the function of PGRP in mammals remained unknown. Because mammals do not have the prophenoloxidase cascade, mammalian PGRP must play another role.

PGN, an essential cell wall component of virtually all bacteria, is especially abundant in Gram-positive bacteria (6). PGN interacts with both humoral (PGP, lysozyme, complement, and soluble CD14) and cellular (hemocytes, macrophages, and lymphocytes) components of the immune system in both vertebrates and invertebrates (7–9). In mammals, PGN activates macrophages through two pattern recognition receptors, CD14 (10–13) and Toll-like receptor-2 (TLR2) (14–16), and induces production of cytokines and chemokines (8–10, 13, 17, 18). Overproduction of these cytokines causes all major clinical manifestations of infections, including fever, inflammation, leukocytosis, hypotension, decreased peripheral perfusion, malaise, sleepiness, decreased appetite, arthritis, and in most severe cases, circulatory shock and multiple organ failure (7–9).

A novel protein, named Tag7, that was expressed in some tumor cell lines, was recently cloned and proposed to be a novel cytokine, a member of the tumor necrosis factor (TNF) family, with TNF-like cytotoxic activity (19). However, because Tag7 has the same sequence as mouse PGRP (5, 19), it is difficult to reconcile the proposed PGN binding and TNF-like cytotoxic activities attributed to the same protein.

Therefore, the aims of this study were to determine the following: (i) the specificity of mammalian PGRP binding; (ii) which cells express PGRP mRNA and protein; (iii) whether PGRP has PGN-lytic, bacteriolytic, and/or bacteriostatic activities; (iv) whether PGRP has TNF-like cytotoxic activity; and...
(v) the effect of PGRP on the main functions of neutrophils and macrophages.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant full-length mouse PGRP (182 amino acids) was expressed in baculovirus-infected SF-9 cells and purified by nickel affinity chromatography as described (5), except that fast protein liquid chromatography was used for the purification and was kept at 4 °C in 10% glycerol. Affinity-purified recombinant PGRP gave one 20-kDa band on 12% SDS-PAGE stained with Coomassie Blue (Fig. 1C). N-terminal amino acid microsequencing (on an Applied Biosystem 476A sequencer) of this PGRP band (after blotting to Immobilon P) revealed the following residues: XSFIY (X is most likely Cys, which cannot be determined by the sequencer), thus confirming the predicted protein sequence of mouse PGRP (5) and demonstrating that the purified recombinant preparation was a mature full-length protein devoid of the signal peptide. PGRP was negative for endotoxin in the Limulus assay (<3 pg of endotoxin/μg of PGRP).

Soluble PGN (sPGN), a polymeric uncross-linked PGN of approximate average M₆, 125,000, released from Staphylococcus aureus Rb or 845 grown in the presence of m Salmonella minnesota R595 (ReLPS, a minimal naturally occurring endotoxic structure of LPS, M₆, 2000–3000), obtained from Sigma, and a disaccharide dipeptide (GlnGlc-β1-4-MDP) were from Calbiochem. No significant endotoxin contamination of these preparations was detected (<1 ng of endotoxin/mg), determined by the Limulus assay (20). *S. aureus* ribitol teichoic acid and lipoteichoic acid (LTA) were the same as before (13).

Rough LPS from Salmonella minnesota Rs 95 (ReLPS, a minimal naturally occurring endotoxic structure of LPS, M₆, 2000–3000), obtained from Sigma, was resuspended in 0.1% Triton X-100, heated at 56 °C, and phenol extracted (Sigma), was dissolved at 2.5 mg/ml in 0.2% triethylamine, and its purity was analyzed as described before (22). Purified smooth LPS, obtained from Escherichia coli O113 by phenol/water extraction (refined endotoxin activity 1.083 g/ml and 1.119 g/liter, Sigma) and were 90% pure as determined by morphology on Wright-stained smears, nonspecific esterase staining, and phagocytosis of latex particles, and did not contain any detectable PMNs (<0.1%).

Spleen cells and thymocytes were obtained by cutting the organs, releasing cells from the capsules, and discarding connective tissues and capsules. Bone marrow cells were obtained by flushing isolated femurs with RPMI 1640 with 5 units/ml heparin, and their spleens, thymuses, and femurs were removed. Peripheral blood mononuclear cells (PBMC) were separated from heparinized blood by centrifugation through Histopaque (density 1.077 g/ml, Sigma) and were 90% pure. Polymorphonuclear leukocytes (PMNs) were separated by centrifugation through two Histopaque layers (density 1.083 g/ml and 1.119 g/liter, Sigma) and were 90% pure as determined by morphology on Wright-stained smears. Peritoneal macrophages were separated from peritoneal washings by adhesion to plastic (23, 24) and were >95% pure as determined by morphology on Wright-stained smears, nonspecific esterase staining, and phagocytosis of latex particles, and did not contain any detectable PMNs (<0.1%).

Human peripheral blood, obtained from healthy males (24–49-year-old Caucasian, Asian, or African American) was isolated by centrifugation through two Histopaque layers (density 1.077 g/ml and 1.119 g/liter, Sigma) into PBMC and into PMNs (98% pure as determined by morphology on Wright-stained smears). Monocytes were isolated from PBMC by adherence to plastic (25, 26) and were >95% pure as judged by morphology on Wright-stained smears, phagocytosis of latex, and nonspecific esterase and did not contain any detectable PMNs (<0.1%). B lymphocytes, T lymphocytes, and NK cells were isolated from PBMC by FACS cell sorting after labeling with phycoerythrin-conjugated anti-human IgG (whole molecule) or FITC-conjugated anti-mouse CD3 monoclonal Ab (Sigma), respectively, and sorting on the FACS-Star Plus cell sorter (Becton Dickinson, San Jose, CA). B and T lymphocytes were >97% pure as determined by FACS analysis and did not contain any detectable PMNs (<0.1%).

**Cells and Cell Lines**—Mice (female 6–16-week-old BALB/c or ICR from Harlan Sprague-Dawley, Indianapolis, IN) were anesthetized with ether and bled from the retro-orbital venous plexus; the cells from their peritoneal cavities were washed out with RPMI 1640 with 5 units/ml heparin, and their spleens, thymuses, and femurs were removed. The following cell lines, all obtained from ATCC (Manassas, VA) and cultured as recommended by ATCC, were used: (a) mouse: B lymphocytic cells, A20, 2PK-3, BCL-1, and WEHI-231; pre-B-cells, 70Z/3; cultured as recommended by ATCC, were used: (a) mouse: B lymphocytic cells, A20, 2PK-3, BCL-1, and WEHI-231; pre-B-cells, 70Z/3; fibroblast, 929 (strain L); and (b) for human: B lymphocytic cells, RAJI; T lymphocytic cells, MOLT-4; monocytic cells, THP-1 and U-937; and fibroblast, HeLa. Human umbilical vein endothelial cells were obtained and cultured as described before (25).

**Expression of PGRP mRNAs by RT-PCR**—Total RNA was isolated from human umbilical vein cells centrifuged at 1000 × g for 10 minutes (Qiagen, Valencia, CA) using mini-spin columns as recommended by the manufacturer. A single tube, two-enzyme Access RT-PCR System (Promega, Madison, WI) was used as recommended by the manufacturer. Brieﬂy, ﬁrst strand cDNA was synthesized in a 500-μl reaction mixture containing 1× avian myeloblastosis virus/TIF reaction buffer, 0.2 mM dNTP, 1 mM MgSO₄, 0.6 units/μl avian myeloblastosis virus reverse transcriptase, 0.1 unit/ml T3 DNA polymerase, 1 μM upstream and

The binding of 125I-PGRP (200 ng/ml) to bacteria, insoluble PGN, and other insoluble preparations was performed as before with the amounts of bacteria or insoluble preparations (used instead of the agarose) included under “Results.”

**Binding of Native PGRP to sPGN and Bacteria**—Mouse bone marrow cells (obtained as described below) were lysed in 0.2 M Tris/HC1, pH 7.0, with 0.2 M NaCl, 4 mM EDTA, 10% glycerol, 1% Nonidet P-40, and a mixture of protease inhibitors. Mouse recombinant PGRP (2 μg/group in the same lysis buffer with 2 mg/ml gelatin) or cell lysates (from 20 × 10⁶ bone marrow cells/group) were incubated for 2 h at 4 °C with 15 μl of sPGN agarose, 250 μg of *M. luteus* cells, control agarose, or latex particles, and centrifuged at 10,000 × g. The sediments were suspended in PAGE sample buffer, and 5× sample buffer was added to the supernatants (1% SDS and 1% 2-mercaptoethanol final concentrations). The samples were boiled and subjected to 12% SDS-PAGE and Western blotting with anti-PGRP antibodies as described below.

The following cell lines, all obtained from ATCC (Manassas, VA) and cultured as recommended by ATCC, were used: (a) mouse: B lymphocytic cells, A20, 2PK-3, BCL-1, and WEHI-231; pre-B-cells, 70Z/3; fibroblast, 929 (strain L); and (b) for human: B lymphocytic cells, RAJI; T lymphocytic cells, MOLT-4; monocytic cells, THP-1 and U-937; and fibroblast, HeLa. Human umbilical vein endothelial cells were obtained and cultured as described before (25).
downstream primers, and 1 μg of total RNA. Samples were amplified in a GeneAmp 9600 thermocycler (Perkin-Elmer). Amplification cycles were preceded by a 2-min 94 °C denaturation. Subsequent stages were at 94 °C for 30 s, 52 °C for 60 s, and 72 °C for 90 s, for the number of cycles indicated under “Results.” The primers for murine and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as a housekeeping gene standard, were (28) as follows: sense, 5′-ACC ACA GTC CAT GCC ATC ACC 3′, and antisense, 5′-TCC ACC ACC CTG TTG ATC TA3′ which yields a 452-bp product. The primers for PGRP, designed based on the mouse and human PGRP sequences (5), were (as follows): mouse sense, 5′-GCA ATG TGC AGC ATT ACC AC3′, and mouse antisense, 5′-ATG TGG TAT CGC ACA C3′ which yields a 358-bp product; human sense, 5′-ATG TGG TAT CGC ACA C3′; and human antisense, 5′-GTC CTT TGA GCA CAT AGT TG 3′ which yields a 342-bp product. PCR products were subjected to 2% agarose gel electrophoresis and visualized by staining with ethidium bromide. 50-bp DNA step-ladder was used as the molecular weight standard. The sequences of all amplified PCR products were confirmed following extraction from the agarose gel and purification using QIAquick PCR purification kit (Qiagen) as recommended by the manufacturer and then using automatic sequencing performed at Genemed Synthesis (South San Francisco, CA).

Genetic Analysis—Mouse PGRP C-terminal (DYW-VIQWSWEHYRE) and N-terminal (RALPSECRSLHC) peptides were synthesized and purified as described (32) for use in the synthetic peptide pool for Mouse PGRN (South San Francisco, CA) and then coupled to keyhole limpet hemocyanin with glutaraldehyde (C-terminal peptide), to bovine serum albumin (BSA) with m-maleimidobenzylox-N-hydroxysuccinimide ester (N-terminal peptide), or to ovalbumin with glutaraldehyde (both peptides) as described (29). Female New Zealand White rabbits were immunized with an emulsion of both peptide-carrier conjugates with keyhole limpet hemocyanin and BSA in Complete Freund’s adjuvant, and were boosted several times with an emulsion of the same peptide conjugates in incomplete adjuvant, followed by boosting with both peptides-ovalbumin conjugates in Complete and then in incomplete Freund’s adjuvants as described (30). The production of antibodies was monitored by Western blots with purified mouse PGRP and anti-rabbit peroxidase-labeled second antibody and enhanced chemiluminescence as described (13). The digests were subjected to SDS-PAGE and blotted onto nitrocellulose membranes and blocked with 5% milk (17). The membranes were incubated with rabbit anti-PGRP serum (1:4000), and immunoreactive bands were detected with anti-rabbit IgG second antibody and ECL as described (13). The bands were detected with anti-rabbit IgG second antibody and ECL as described (13).

Distribution of PGRP Expression by Western Blot—Mouse or human cells (106/group) were dissolved in 100 μl of lysis buffer (0.1% TritonX, pH 8.0, with 0.1 mM NaCl, 4 mM EDTA, 30% glycerol, 1% Nonidet P-40, and a mixture of protease inhibitors), boiled with PAGE sample buffer (2% SDS and 1% 2-mercaptoethanol), and centrifuged at 12,000 × g.

Oxidative Burst (NBT Test)—Bacterially induced oxidative burst in human neutrophils was measured by nitro blue tetrazolium (NBT) reduction method (33) using Sigma clinical diagnostic kit as recommended by Sigma. Briefly, stimulants (M. luteus, B. subtilis, E. coli, LPS, or phosphatidylinerse (PDB) + ionomycin) were preincubated in siliconized glass vials for 10 min at 37 °C in medium alone or with PGRP (10 μl), and then 50 μl of NBT solution and 50 μl of fresh human peripheral blood with 10 units/ml of heparin were added to yield 40 μg/ml bacteria, 1 μg/ml LPS, 100 ng each of PDB and ionomycin, and 1, 3, 10, or 10 μg/ml PGRP (final concentrations). The vials were incubated for 10 min at 37 °C and for an additional 10 min at 22 °C. Blood smears were prepared and stained with the Wright stain, and the numbers of neutrophils that contained or did not contain the NBT granules were counted under the microscope.

Induction of TNF-α and IL-6—Mouse macrophage RAW264.7 cells grown in serum-free medium were adapted to growth in serum-free HL-1 medium (BioWhittaker, Walkersville, MD) were cultured and stimulated as before (17) with medium alone (Nil), or with 0.1, 1, or 10 ng/ml SPGN, or with 0.1 μg/ml serum-free conditions or 0.01 μg/ml (medium with serum) ReLPS, without or with 1 or 10 ng/ml PGRP. The culture supernatants were collected after 6 h of stimulation at 37 °C, 5% CO2 and assayed for TNF-α concentrations using the L929 cytotoxicity assay (17) described below or for IL-6 using the IL-6-dependent TD71 cells as before (25).

Cytotoxicity for Mammalian Cells—To test if PGRP has any TNF-like cytotoxic activity, we used the standard L929 cell cytotoxicity assay that is commonly used for measuring cytotoxic activity of TNF, performed as described (17). Briefly, medium alone, serial double dilutions of PGRP alone (20 μg/ml to 10 μg/ml), or recombinant mouse TNF-α standard alone (specific activity, 4 × 10^7 units/mg, from Genzyme, Boston), or of combinations of PGRP and TNF-α were added to L929 cells and incubated for 18 h in the presence of 1.33 μg/ml actinomycin D. The cell morphology was evaluated under a phase-contrast microscope; the number of surviving cells was measured spectrophotometrically after staining with crystal violet, and a standard curve for TNF-α cytotoxicity was fitted into a sigmoidal equation as before (17).

Chemotaxis—Chemotaxis was evaluated as described (34). Medium (RPMI 1640 with 10 μM HEPES, pH 7.2, and 1 or 10 mg/ml BSA), sPGN alone, PGRP alone, or sPGN and PGRP together (at 0.001 to 1 μg/ml), or control chemotactants, 0.1 nM formyl-methyl-leucyl-methionine (FMLP, Sigma), 20 ng/ml monocyte chemotactic protein-1 (MCP-1, specific activity 100 units/μg), or 200 ng/ml GRO-α (specific activity 100 units/μg) partially purified from human recombinant E. coli and obtained from the National Cancer Institute, Fredrick, MD), were placed in the wells of the 96-well disposable ChemoTx chemotactic plates (Neuroprobe, Gaithersburg, MD). Human monocytes or PMNs were isolated as described above under “Cells and Cell Lines.” Monocytes were allowed to phagocytize latex particles (for 30 min at 37 °C, 5% CO2 phagocytosis of latex had no adverse effect on
the ability of the monocytes to migrate to chemotactic stimuli but greatly aided their identification and differentiation from other mono-nuclear cells), were washed with medium, and 0.48 × 10⁶ cells (in 80 μl) were put on top of filters with 8-μm pores placed over the chambers containing the chemotactic stimuli. The chambers were incubated for 90 min at 37 °C, 5% CO₂, the filters were washed with PBS, dried, and stained with Wright’s stain, and the numbers of monocytes that migrated into the filter were counted on equal numbers of random fields stained with Wright’s stain, and the numbers of monocytes that migrated into the filter were counted on equal numbers of random fields under an oil immersion microscope (34). PMNs (0.18 × 10⁶ in 60 μl) were put on top of filters with 5-μm pores placed over the chambers containing the chemotactic stimuli. The chambers were incubated for 30 min at 37 °C, 5% CO₂. The cells were scraped off the filters (without separating the filters from the chambers); the filters were washed with PBS and incubated with 40 μl of 10 mM EDTA for 30 min at 4 °C; the plates were centrifuged at 1500 rpm for 10 min at 4 °C, and PMNs in the lower chamber were counted.

RESULTS

PGRP Binds Peptidoglycan and Gram-positive Bacteria with Nanomolar Affinity—To determine the specificity, kinetics, and affinity of interaction between mouse PGRP and PGN, we developed a binding assay, similar to the binding assay that was used to demonstrate the binding of PGN to CD14 (13), in which binding of ¹²⁵I-labeled PGRP to soluble PGN (sPGN) immobilized on agarose beads was studied. Mouse PGRP bound to sPGN-agarose but not to control agarose or ReLPS-agarose, in a dose-dependent manner (Fig. 1A), and maximum binding was reached in less than 10 min (Fig. 1B). The ratio of specific to nonspecific binding was 50 to 150 (Fig. 1).

PGRP binding was specific for polymeric sPGN (Fig. 2), as evidenced by the following: (a) binding of PGRP to sPGN-agarose and no binding to control agarose; (b) inhibition of binding by sPGN with an IC₅₀ = 0.2 μg/ml (2 μM); (c) no binding of PGRP to low M₉ GPG components (MDP, GlcNac-MDP, or PGN pentapeptide) conjugated to agarose; (d) more than 10–20 times lower binding to ReLPS and smooth LPS; (e) no competitive inhibition by low M₉ PGN components (GlcNac-MDP, and also by MDP, GlcNac, and pentapeptide [not shown]); and (f) 3–4 orders of magnitude less inhibition by smooth LPS, ReLPS, LTA, or ribitol teichoic acid. Of note, the same batches of sPGN and by the enhancing effect of unlabeled PGRP on the binding of ¹²⁵I-PGRP to sPGN and by the enhancing effect of unlabeled PGRP on the binding of ¹²⁵I-PGRP to sPGN (see Fig. 4, below). Moreover, it was also suggested that Tag7 (PGRP) forms aggregates in solution (19).

At low concentrations (0.005–0.125 μg/ml), PGRP bound to sPGN with Kₐ = 13 nM (Fig. 3). At higher concentrations (0.125–1 μg/ml), PGRP showed cooperative binding to sPGN, indicated by concave upwards Scatchard plot, with a second low affinity binding (Kₛ = 70 nM). This cooperative binding suggests aggregation of PGRP at higher concentrations in the presence of PGN or binding of multiple molecules of PGRP to each PGN molecule. This hypothesis of cooperative binding was also supported by the inability of unlabeled PGRP at higher concentrations to inhibit competitively binding of ¹²⁵I-PGRP to sPGN and by the enhancing effect of unlabeled PGRP on the binding of ¹²⁵I-PGRP to sPGN (see Fig. 4, below). Moreover, it was also suggested that Tag7 (PGRP) forms aggregates in solution (19).

PGRP binding to sPGN was not inhibited by sCD14 (Fig. 4) or by the 1–152 N-terminal amino acid fragment of sCD14, and binding of ³²P-sCD14 to sPGN was not inhibited by PGRP (data not shown). These results suggest that PGRP and sCD14 bind to different epitopes on sPGN.

PGRP binding to sPGN was inhibited by lysozyme, which binds to and hydrolyzes a bond in the (GlcNac-MurNAc)₃ fragment of the PGN glycan chain, but not by lysisostaphin, which binds to and hydrolyzes the peptide portion of PGN (Fig. 4). These results suggest that PGRP binds to the similar epitope on the glycan chain of PGN as lysozyme.

Mouse PGRP bound to some Gram-positive bacteria (Micrococcus and Bacillus), and this binding was 4–8 times higher than to S. aureus (another Gram-positive bacterium) or E. coli (a Gram-negative bacterium) (Fig. 5A). Based on the amount of PGN in Micrococcus and Bacillus cells, binding of PGRP to these bacteria was approximately 10 times lower than the binding to sPGN-agarose (there is 1.5 μg of sPGN bound per 1 μl of sPGN-agarose, and approximately 1 μg of PGN/10–20 μg of Gram-positive bacteria, Ref. 35). This lower binding of PGRP to PGN in intact bacteria may be related to more limited accessibility for PGRP binding of the peptide-cross-linked PGN

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R. Dziarski, unpublished data.
Peptidoglycan Recognition Protein

Fig. 2. $^{125}$I-PGRP specifically binds to sPGN but not to other bacterial polymers. A, binding of $^{125}$I-PGRP (200 ng/ml) to 0.5 μl of sPGN-agarose or to the indicated agarose conjugates was measured as in Fig. 1A. The results are means ± S.E. of three experiments. B, competitive inhibition of binding of $^{125}$I-PGRP (200 ng/ml) to 0.5 μl of sPGN-agarose in the absence (control) or presence of various concentrations of the indicated competitors was measured, and the results (means from three experiments) were calculated as the percent of control binding without a competitor; the S.E. were less than 15% and are not shown.

![Graph](image1)

**Fig. 3.** PGRP binds to sPGN with nanomolar affinity. A, increasing concentrations of $^{125}$I-PGRP were incubated with 0.5 μl of sPGN-agarose or control agarose, and the amounts of $^{125}$I-PGRP bound to agarose and remaining unbound (free) were measured. Non-specific binding was the amount of $^{125}$I associated with control agarose; specific binding was total amount of $^{125}$I associated with sPGN-agarose minus non-specific binding. The results are means of three experiments. B, the Scatchard plot was fitted using Cricket Graph software, and mean apparent dissociation constants ($K_d$) ± S.E. were calculated using curve fitting to a hyperbolic function (SigmaPlot software).

![Graph](image2)

**Fig. 4.** PGRP cooperatively binds to sPGN and PGRP binding is inhibited by lysozyme. Competitive inhibition of binding of $^{125}$I-PGRP (200 ng/ml) to 0.5 μl of sPGN-agarose by various concentrations of the indicated PGN-binding proteins was measured, and the results (means from three experiments) were calculated as the percent of control binding without a competitor; the S.E. were less than 15% and are not shown.

![Graph](image3)

incorporated into the cell wall. Still lower binding of PGRP to E. coli and S. aureus suggests even poorer availability of the form of PGN that binds PGRP on the surface of E. coli, in which PGN is hidden below the outer membrane, and S. aureus, in which PGN is highly cross-linked and heavily modified by covalently linked ribitol teichoic acid, proteins, and O-acetylation (36).

PGRP also binds to insoluble S. aureus cell wall PGN, zymosan (Saccharomyces cell wall), and chitin but did not bind to cellulose (Fig. 5B). However, this binding was rather poor, since 300–600 times less PGRP bound to insoluble cell wall PGN than to soluble agarose-bound PGN from the same S. aureus strain. The binding to zymosan and chitin was even lower than to insoluble PGN. The two main differences between the soluble and insoluble PGN are extensive peptide-cross-linking in insoluble PGN (which could make PGRP-binding sites unavailable) and extensive O-acetylation in insoluble cell wall PGN, in contrast to sPGN, which is not cross-linked and not O-acetylated. If PGRP shows preference for non-O-acetylated PGN, this specificity would be similar to the specificity of lysozyme, which only acts on non-O-acetylated PGN (which makes S. aureus resistant to lysozyme, Ref. 36). These results are consistent with the binding to intact bacteria (Fig. 5A), because Micrococcus and Bacillus, which bind PGRP much better than S. aureus, are, in contrast to S. aureus, also highly sensitive to lysozyme, because they have non-O-acetylated PGN (36). Therefore, all these data together suggest that PGRP has a similar specificity to lysozyme but 200–300 times higher affinity (37).

Native mouse PGRP had a similar binding potential as the recombinant PGRP, as shown by similar binding of virtually all native PGRP from the lysates of mouse bone marrow cells and recombinant PGRP to sPGN-agarose and Micrococcus, and no binding of either PGRP to control agarose or latex particles, as detected by Western blotting (Fig. 6). These results confirm that the recombinant PGRP has the proper conformation for the binding to PGN and bacteria and that the C-terminal His$_6$ tag present in the recombinant PGRP has no major influence on the binding of PGRP to PGN and bacteria.

**Fig. 5.** PGRP cooperatively binds to sPGN and bacteria and that the C-terminal His$_6$ tag present in the recombinant PGRP has no major influence on the binding of PGRP to PGN and bacteria.

![Graph](image4)

**Fig. 6.** Western blotting of PGRP and bacterial PGN. A, blotting of indicated bacterial PGN and recombinant PGRP with anti-PGRP antibody. B, blotting of recombinant PGRP with anti-PGRP antibody. The results are consistent with the binding of either PGRP to control agarose or latex particles, as shown by similar binding of virtually all native PGRP from the lysates of mouse bone marrow cells and recombinant PGRP to sPGN-agarose and Micrococcus, and no binding of either PGRP to control agarose or latex particles, as detected by Western blotting (Fig. 6). These results confirm that the recombinant PGRP has the proper conformation for the binding to PGN and bacteria and that the C-terminal His$_6$ tag present in the recombinant PGRP has no major influence on the binding of PGRP to PGN and bacteria.

**Fig. 7.** PGRP cooperatively binds to sPGN and PGRP binding is inhibited by lysozyme. Competitive inhibition of binding of $^{125}$I-PGRP (200 ng/ml) to 0.5 μl of sPGN-agarose by various concentrations of the indicated PGN-binding proteins was measured, and the results (means from three experiments) were calculated as the percent of control binding without a competitor; the S.E. were less than 15% and are not shown.

**Fig. 8.** Western blotting of PGRP and bacterial PGN. A, blotting of indicated bacterial PGN and recombinant PGRP with anti-PGRP antibody. B, blotting of recombinant PGRP with anti-PGRP antibody. The results are consistent with the binding of either PGRP to control agarose or latex particles, as shown by similar binding of virtually all native PGRP from the lysates of mouse bone marrow cells and recombinant PGRP to sPGN-agarose and Micrococcus, and no binding of either PGRP to control agarose or latex particles, as detected by Western blotting (Fig. 6). These results confirm that the recombinant PGRP has the proper conformation for the binding to PGN and bacteria and that the C-terminal His$_6$ tag present in the recombinant PGRP has no major influence on the binding of PGRP to PGN and bacteria.
Fig. 5. PGRP binds to bacteria, insoluble cell wall PGN, zymosan, and chitin. 125I-PGRP (200 ng/ml) and 25 µg of the indicated bacteria or cellulose (A) or the indicated amounts of insoluble S. aureus cell wall PGN, zymosan, chitin, or cellulose (B) were incubated for 10 min at 37 °C and centrifuged through 0.8 M sucrose, and the amount of 125I bound to the pellet was measured. The results are means of four samples from two experiments. B, the S.E. were less than 15% and are not shown.

Macrophage, one pre-B lymphocyte, and one plasma cell (hybridoma) cell lines (Fig. 7B). This low level expression of PGRP mRNA in B cell tumors is not surprising, because Tag7 (identical to PGRP) was expressed in some tumors and was cloned from a tumor cell line (19). PGRP mRNA expression in B cell tumors may be related to its chromosomal location in band 7A3, because this band is genetically linked with lupus nephritis in MRL and New Zealand mice, which are characterized by over-activation of B cells (19).

Stimulation of T or B lymphocytes, macrophages, B lymphocytic, pre-B cell, or macrophage cell lines with PGN, LPS, or Gram-positive bacteria for 6–72 h did not induce PGRP expression in these cells (data not shown). Weak expression of PGRP mRNA in mouse spleen cells and PBMC was likely due to low level of contamination of these cells with PMNs (because, in contrast to humans, the density of mouse PMNs is very similar to the density of mononuclear cells, and differential centrifugation through Ficoll is not as effective in separating mouse PMNs from mononuclear cells). 3

In humans, PGRP mRNA was also strongly expressed in PMNs and was not expressed in PBMC, monocytes, B lymphocytes, T lymphocytes, and NK cells (Fig. 7C). PGRP mRNA was also not expressed in human T lymphocyte (MOLT-4), B lymphocyte (RAJI), and monocytic (U-937 and THP-1) cell lines (Fig. 7C).

Although the expression of most proteins is regulated at the transcriptional level, expression of some proteins is regulated at the translational level, and the presence of PGRP mRNA does not necessarily mean the expression of PGRP protein. Moreover, the high sensitivity of PCR-based methods makes them susceptible to false-positive results from contamination of non-expressing cells by small numbers of expressing cells. For these reasons, we next determined the expression of PGRP protein in mouse cells using Western blots and anti-mouse PGRP antibodies.

PGRP protein was exclusively expressed in mouse PMNs and bone marrow cells and was not expressed in spleen cells, PBMC, purified B lymphocytes, purified T lymphocytes, and purified peritoneal macrophages (Fig. 8A). The anti-PGRP Abs used for detection of PGRP were specific for PGRP, because the appearance of only the PGRP band (but not other nonspecific bands) on the Western blots was inhibited by the PGRP peptides (Fig. 8B). The appearance of the PGRP band was not inhibited by an unrelated control peptide (not shown). The amount of PGRP in neutrophils was approximately 1–2 µg of PGRP/10^6 PMNs, as estimated on Western blots by comparing serial dilutions of cell lysates and purified PGRP (data not shown). PGRP protein was also not detectable in any of the mouse B lymphocytic, T lymphocytic, pre-B cell, hybridoma, or macrophage cell lines (data not shown). These results indicate exclusive expression of PGRP protein in PMNs. The expression of PGRP in the bone marrow is likely due to the high content of PMNs in the bone marrow, which is the site of PMN production and maturation and contains the reserve pool of PMNs.

PGRP Is Not a PGN-lytic Enzyme, but It Inhibits Growth of Gram-positive Bacteria—We next tested whether mammalian PGRP had a PGN-lytic or bacteriolytic activity because of three reasons. First, because PGRP sequence has some homology to the T3 and T7 bacteriophage lysozymes (5), which are amidases (i.e. they hydrolyze the bond between muramic acid and the peptidoglycan), unlike the vertebrate lysozymes, which is a muramidase, i.e. it hydrolyzes the glycosidic bond between GlcNAc and MurNAc of the glycan chain). Second, because binding specificity of PGRP to PGN is similar to vertebrate lysozyme. And third, because PGRP is expressed in PMNs, and one of the functions of PMNs is digestion of phagocytized bacteria.

However, mouse PGRP that bound to PGN with nanomolar affinity did not have any detectable PGN-lytic activity even at 100 µg/ml, when incubated for 48 h at 37 °C. By contrast, under the same conditions, complete lysis of sPGN with control enzymes, lysozyme, and lysostaphin (determined by Western blots) was detectable at 0.5 µg/ml of lysozyme and 5 µg/ml of lysostaphin, and 50% digestion was detectable at 0.05 and 0.5 µg/ml, respectively.

PGRP also had no detectable bacteriolytic activity for M. luteus at concentrations up to 25 µg/ml when incubated for up to 24 h. By contrast, lysozyme caused virtually complete lysis of the bacteria (reduction of A405 from 0.75 to 0.05) in 30 min at 10 µg/ml and in 90 min at 1 µg/ml lysozyme.

We next considered the possibility that PGRP may have an

\(^3\) C. Liu and R. Dziarski, unpublished data.
antibacterial effect (bacteriostatic or bactericidal), because PGRP is present at high concentrations in PMNs, and because the main function of PMNs is phagocytosis and killing of bacteria. Indeed, PGRP significantly inhibited the growth of Gram-positive bacteria (S. hemolyticus and B. megaterium, Fig. 9, B–D, and also S. warneri and S. capitis, not shown) but had no effect on the growth rate of Gram-negative bacteria (E. coli, Fig. 9A, and also E. cloacae, not shown). PGRP by itself, however, was not bactericidal, and it only inhibited the growth rate of bacteria by about 50%, i.e. it took approximately twice as long to generate similar numbers of bacteria in the cultures with PGRP compared with cultures without PGRP (Fig. 9D and data not shown). Upon prolonged incubation, both cultures eventually reached similar numbers of bacteria (Fig. 9D and data not shown).

PGRP Inhibits Phagocytosis and Oxidative Burst in Leukocytes—We next tested the effect of PGRP on phagocytosis of bacteria and on bacterially induced oxidative burst in leukocytes, because PGRP could be present in serum at low concentrations and/or it could be released from PMNs at the inflammatory sites (the concentration of PGRP in mouse serum was lower than 5 μg/ml, determined on Western blots). Because of its nanomolar affinity of binding to PGN and bacteria, PGRP (released from PMNs) even at low concentrations could modulate the host responses to PGN and bacteria by analogy to complement components or mannose-binding proteins that act as opsonins (1, 2) or to LPS-binding protein that enhances cell responses to LPS (38, 39).

First, we evaluated the effect of PGRP on the induction of TNF-α and IL-6 production by a mouse macrophage cell line,
Peptidoglycan Recognition Protein

RAW264.7, adapted to growth in serum-free medium. Serum-free conditions were selected to avoid any possible interference of the native PGRP that could be present at low concentration in the serum. PGRP, at 10 μg/ml, inhibited sPGN-induced TNF-α and IL-6 production by 62 and 80%, respectively, but did not significantly inhibit ReLPS-induced production of TNF-α and IL-6 (Fig. 11). At 1 μg/ml, PGRP still inhibited sPGN-induced production of IL-6 by 65%, but it did not significantly inhibit the production of TNF-α (not shown).

Next we tested the influence of serum on the effect of PGRP on the sPGN-induced cytokine production, because serum could contain other components that could either enhance or inhibit the effect of PGRP. The sPGN- and ReLPS-induced production of TNF-α and IL-6 in RAW264.7 cells cultured in the presence of 10% serum was not significantly changed by the presence of 1 or 10 μg/ml of PGRP, thus demonstrating that serum abolished the inhibitory effect of PGRP on sPGN-induced cytokine production. This may be due to the already diminished responses of cells to sPGN in the presence of serum that we have observed before (14), although the components in the serum responsible for this decreased responsiveness to sPGN are still unknown.

**PGRP Has No TNF-like Cytotoxicity—** We next tested whether PGRP was cytotoxic for tumor cell lines, because it was suggested that Tag7, which has identical sequence to PGRP (5), is a new cytokine with a TNF-like cytotoxicity (19). However, none of our mouse PGRP preparations (which all bound PGN with nanomolar affinity, see above), at 20 pg/ml to 10 μg/ml, had any cytotoxicity for L929 cells under the conditions at which TNF-α induced 50% cytotoxicity at 10 pg/ml and 95% cytotoxicity at 100 pg/ml, as evaluated microscopically and measured spectrophotometrically (Fig. 12). Moreover, PGRP had no effect on TNF-α-induced cytotoxicity for L929 cells (Fig. 12), and it also was not cytotoxic for three other tumor cell lines and for primary cells (mouse and human leukocytes, human fibroblasts, and primary human endothelial cells). This lack of PGRP cytotoxicity was confirmed independently in two laboratories.

**PGRP and PGRP-sPGN Complexes Are Not Chemotactic—** Because chemotaxis and production of chemotactic chemokines are the most prominent host responses to bacteria and bacterial products, including PGN (18), we also tested if PGRP by itself or in combination with sPGN had any chemotactic activity. However, neither PGRP nor sPGN by themselves or together (at 0.001 to 1 μg/ml) were chemotactic for human monocytes or PMNs, whereas, in the same experiments, control chemotactic agents induced 8–14-fold increases in directional migration of monocytes (0.1 nM fMLP and 200 ng/ml GRO-α) and neutrophils (0.1 nM fMLP and 200 ng/ml GRO-α).

**DISCUSSION**

Our results demonstrate that mammalian PGRP binds to PGN and Gram-positive bacteria (with unmodified PGN) with nanomolar affinity, is expressed in neutrophils, inhibits growth of Gram-positive bacteria, and inhibits some of the bacterially induced functions of neutrophils and macrophages. These results support the notion (5) that mammalian PGRP has analogous function to insect PGRP (4, 5, 42), i.e. it most likely plays a role in antibacterial innate immunity. However, the exact
function of PGRP in mammalian and insect immunity may be different, because in mammals PGRP may function as an antibacterial intracellular protein present in neutrophils, whereas in insects PGRP is likely to function in a humoral defense mechanism (4, 42).

It was suggested that PGRP is a pattern-recognition protein for PGN (5, 42), as it can recognize PGN, a cell wall component common to all bacteria. CD14 is another pattern-recognition protein that also functions to recognize PGN. However, the specificities, the structures, and the functions of PGRP and CD14 are quite different. The specificity of PGRP is much narrower than that of CD14; PGRP binds with high affinity only to soluble, uncross-linked, and unmodified polymeric PGN and binds much poorer to highly peptide cross-linked O-acetylated PGN and does not bind to low M<sub>r</sub> soluble or solid-phase-bound PGN fragments and other bacterial cell wall components, such as LTA or LPS. By contrast, sCD14 binds to all of these compounds (except for soluble low M<sub>r</sub> PGN subunits) with similar affinity (13). Also, PGRP binds to PGN with a faster kinetics and a higher affinity than CD14. Moreover, PGRP and CD14 seem to be specific for different epitopes on PGN, because PGRP and CD14 do not compete with each other for binding to PGN. PGRP is expressed exclusively in PMNs, where it is present in granules and probably functions as an intracellular antibacterial protein, whereas CD14 is a cell-surface receptor expressed primarily on monocytes and macrophages that triggers pro-inflammatory responses of these cells (8–13). In addition, CD14 is present in a soluble form in plasma, where it functions to activate CD14-negative cells and to enhance the responses of CD14-positive cells (8–13). By contrast, PGRP inhibits some of the cell-activating effects of PGN and Gram-positive bacteria. Therefore, given all these differences in the specificities and functions of PGRP and CD14, it is not surprising that they do not share any structural or sequence homology. PGRP also has no structural and sequence homology to PGRP-LTA or LPS. By contrast, sCD14 binds to all of these PGN fragments and other bacterial cell wall components, such as LTA or LPS. However, two aspects of our results on the expression and function of PGRP differ from the results reported for Tag7 (19).

First, we found that PGRP is primarily expressed in neutrophils, is only very weakly expressed in spleen cells, and is not expressed in purified macrophages, T and B lymphocytes, and NK cells that are free of neutrophils. Tag7 mRNA was shown to be present in peritoneal macrophages, spleen, and lung (19); however, its presence in neutrophils or highly purified lymphocyte subpopulations was not studied (19). We have found that contamination of tissues with blood-derived neutrophils makes these tissues positive on PGRP mRNA expression tests, which could be one possible explanation of the positive results of Tag7 expression in peritoneal macrophages, spleen, and lung (19). Also, in contrast to the results reported for Tag7 (19), we could not detect any increase in PGRP expression in lymphocytes or macrophages following stimulation with PGN, LPS, or bacteria. Thus, our current results are consistent with previously reported (5) very high expression of PGRP in the bone marrow (the site of neutrophil production and of the neutrophil bone marrow reserve pool) and very low expression in other lymphoid tissues, which might have been due to contamination of these tissues with blood. This interpretation is consistent with the expression of Tag7, which could only be detected in freshly isolated spleen cells but not in B and T lymphocytic cell lines (19). It is also possible, however, that some cells other than neutrophils express very low levels of PGRP mRNA and do not express, or express only very low levels of PGRP protein (undetectable by Western blot).

Second, in contrast to Tag7 (19), our PGRP has no TNF-like cytotoxicity for L929 cells and other tumor cell lines, even at concentrations of PGRP 10<sup>6</sup> times higher than the cytotoxic concentrations of TNF-α. The reason for this discrepancy is unclear, although it may be related to the fact that we have used purified PGRP protein, whereas previously crude culture supernatants containing Tag7, rather than purified Tag7 protein, were tested (19). Therefore, our results firmly establish PGRP as a peptidoglycan-binding protein that interacts with bacteria and has no TNF-like cytotoxicity. Also consistent with PGN binding activity of PGRP and its expression in neutrophils is our finding that PGRP inhibits growth of Gram-positive bacteria <em>in vitro</em>. PGRP also has no effect on the cytotoxicity of TNF-α, but it inhibits some of the bacterially induced functions of neutrophils and macrophages, such as phagocytosis of Gram-positive bacteria by macrophages, induction of oxidative burst by Gram-positive bacteria in neutrophils, and induction of cytokine production by PGN in macrophages. This inhibitory effect does not seem to be due to inhibition of PGN binding to CD14, because PGRP and CD14 do not inhibit each others’ binding to PGN, but it could possibly be due to inhibition of activation through other PGN recognition systems, such as TLR2 (14–16). However, the significance of these effects of PGRP <em>in vivo</em> still needs to be elucidated, because PGRP is mostly located intracellularly and because some of these effects (inhibition of cytokine production) were only observed in serum-free but not in serum-

<sup>4</sup> M. C. Nehls and R. Dziarski, unpublished data.
containing medium. These inhibitory effects of PGRP are reminiscent of the inhibitory effects of other antibacterial intracellular neutrophil proteins, such as bactericidal permeability-inducing protein, whose main function is cytotoxicity to Gram-negative bacteria inside phagolysosomes, but which, when used as soluble extracellular proteins, also inhibit cell activation by Gram-negative bacteria and LPS (40, 41).

In summary, mammalian PGRP binds to PGN and Gram-positive bacteria (with unmodified PGN) with nanomolar affinity, is expressed in neutrophils, and inhibits growth of Gram-positive bacteria (40, 41).

In summary, mammalian PGRP binds to PGN and Gram-negative bacteria and LPS (40, 41).

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