Human Peptidoglycan Recognition Protein-L Is an N-Acetylmuramoyl-L-alanine Amidase*

Received for publication, July 17, 2003, and in revised form, August 25, 2003
Published, JBC Papers in Press, September 23, 2003, DOI 10.1074/jbc.M307758200

Zheng-Ming Wang‡§, Xinna Li‡§, Ross R. Cocklin§, Minhua Wang‡, Mu Wang‡, Koichi Fukase†, Seiichi Inamura‡, Shoichi Kusumoto, Dipika Gupta‡, and Roman Dziarski‡**

From the ‡Northwest Center for Medical Education, Indiana University School of Medicine, Gary, Indiana 46408, the §Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana 46202, and the ¶Department of Chemistry, Graduate School of Science, Osaka University, Osaka 560-0043, Japan

Peptidoglycan recognition proteins (PGRPs) are pattern recognition molecules coded by up to 13 genes in insects and 4 genes in mammals. In insects PGRPs activate antimicrobial pathways in the hemolymph and cells, or are peptidoglycan (PGN)-lytic amidases. In mammals one PGRP is an antibacterial neutrophil peptide. We report that human PGRP-L is a Zn2+-dependent N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28), an enzyme that hydrolyzes the amide bond between MurNAc and L-Ala of bacterial PGN. Human PGRP-L has no direct bactericidal activity. The other members of the human PGRP family, PGRP-1, PGRP-1′, and PGRP-S, do not have the amidase activity. The C-terminal region of PGRP-L, homologous to bacterial and bacterial amidases, is required and sufficient for the amidase activity of PGRP-L, although its activity (in the N-terminal Δ1–343 deletion mutant) is reduced. The Zn2+ binding amino acids (conserved in PGRP-L and T7 amidase) and Cys-419 (not conserved in T7 amidase) are required for the amidase activity of PGRP-L, whereas three other amino acids, needed for the activity of T7 amidase, are not required for the activity of PGRP-L. These amino acids, although required, are not sufficient for the amidase activity, because changing them to the “active” configuration does not convert PGRP-L into an active amidase. In conclusion, human PGRP-L is an N-acetylmuramoyl-L-alanine amidase and this function is conserved in prokaryotes, insects, and mammals.

Peptidoglycan recognition proteins (PGRPs)† are pattern recognition molecules that are conserved from insects to mammals and recognize bacteria and their unique cell wall component, peptidoglycan (PGN). Fruit fly (Drosophila melanogaster) has 13 PGRP genes that are transcribed into at least 17 proteins (1–3), and a mosquito (Anopheles gambiae) has 7 PGRP genes that are transcribed into at least 9 proteins (2). Based on the predicted structures of the gene products, insect PGRPs have been grouped into two classes: short PGRPs (PGRP-S), which are small extracellular proteins (19–20 kDa), and long PGRPs (PGRP-L), which have long transcripts and are either intracellular or membrane-spanning proteins (1, 2).

Insect PGRPs have at least five known functions. Silkworm PGRP-S, Drosophila PGRP-L, and probably other PGRPs recognize PGN and bacteria and initiate activation of the prophenoloxidase cascade, which generates antimicrobial melanin (4, 5). Drosophila PGRP-SA, PGRP-SC, and PGRP-LC activate two different pathways that induce production of antimicrobial peptides. PGRP-SA interacts with the lysine-containing PGN from Gram-positive bacteria and activates proteases that generate Spätzle, which activates Drosophila Toll receptor (6–8). Activation of Toll receptor induces production of drosomycin and other antimicrobial peptides that are mainly active against Gram-positive bacteria and fungi (6, 7). PGRP-LC and PGRP-LE recognize the diaminopimelic acid-containing PGN from Gram-negative bacteria and Gram-positive bacilli and activate the “Imd” pathway that is Toll-independent and induces production of diptericin and other antimicrobial peptides that are primarily active against Gram-negative bacteria (3, 8–11). Drosophila PGRP-LC probably also functions in phagocytosis of Gram-negative bacteria (11). Finally, Drosophila PGRP-SC1b is a PGN-lytic enzyme, N-acetylmuramoyl-L-alanine amidase (12).

Mammals have four PGRPs, designated in humans PGRP-S, PGRP-L, PGRP-1, and PGRP-1′ (13, 14). Human PGRPs are differentially expressed in various tissues and organs: PGRP-S mainly in the bone marrow and PMNs, PGRP-L mainly in the liver, and PGRP-1 and PGRP-1′ in the esophagus (13–15). The function of only one mammalian PGRP, PGRP-S, is currently known. PGRP-S is an antibacterial peptide stored in the secretory granules of PMNs (16, 17). It is bacteriostatic or bactericidal (15–17) and functions in intracellular killing of bacteria in PMNs (16). The functions of PGRP-L, PGRP-1, and PGRP-1′ are not known.

The C-terminal regions of all insect and mammalian PGRPs are highly conserved (14). This region also has ~18% identity and ~33% similarity to bacteriophage T7 lysozyme, which is an N-acetylmuramoyl-L-alanine amidase, an enzyme that hydrolyzes the amide bond between the N-acetylmuramic acid and L-alanine of PGN (12, 18–20). T7 lysozyme is a Zn2+-containing protein, and Zn2+ is required for its amidase activity (18). T7 lysozyme amino acids are required for its Zn2+ binding and amidase activity, and at least three additional amino acids are also required for the T7 amidase activity (18). Out of 36 currently known insect and mammalian PGRPs, 12 PGRPs have...
all 4 of these Zn$^{2+}$-binding amino acids conserved, and the remaining 3 amino acids are also either conserved or have permissible substitutions, and, thus are predicted to have amidase activity (Ref. 12 and this report). One of them, Drosophila PGRP-SC1b, was recently shown to be an N-acetylmuramoyl-

\[ \text{L-} \text{alanine amidase} \] (12). Out of the four mammalian PGRPs, only PGRP-L has all of the above amino acids conserved and, therefore, it is predicted to be an amidase (12). The objective of this study was to test this hypothesis and thus, identify the function of human PGRP-L.

**EXPERIMENTAL PROCEDURES**

**PGRP Constructs and Proteins**—Human PGRP-S, PGRP-L, PGRP-1o, and PGRP-Ij and mouse PGRP-S and PGRP-L were cloned in pcDNA3.1 and expressed in COS-7 cells as described previously (14). Human PGRP-L deletion mutants were generated by PCR with the primers listed in Table I. Single amino acid substitution mutants of human PGRP-L, human PGRP-S, and mouse PGRP-S were generated using the site-directed mutagenesis kit from Clontech (Palo Alto, CA) with the primers listed in Table I. All constructs were subcloned into pcDNA3.1 with C-terminal V5 and 6xHis tags and analyzed by Western blotting and by sequencing as described previously (14). Reconstituted PGRP proteins were obtained from transiently transfected COS-7 cell lysates (PGRP-L, PGRP-1o, and PGRP-Ij) or lysates and supernatants (PGRP-S) (14) by affinity chromatography using His-Bind kit (Novagen, Madison, WI) as recommended by the manufacturer, with binding, washing, and elution buffers all containing 20 m\( \text{M} \) Tris (pH 7.9), 0.5 m\( \text{M} \) NaCl, 1% Triton X-100, and 5 mM, 20 mM or 300 mM imidazole, respectively. The proteins were stored in the elution buffer with 10% glycerol at −80 °C.

**PGN Digestion and Amidase Assays**—Soluble uncross-linked PGN was purified by vancomycin-affinity chromatography from *Staphylococcus aureus* Rb grown in the presence of penicillin G and analyzed as described previously (21). PGN was labeled with biotin on the N-terminal glycine of its interpeptide bridge, and its hydrolysis was measured as described previously (22). Complete loss of the biotin from PGN was incubated for 3 days at 37 °C with 250 ng of PGRP-L or in buffer alone (negative control) in 200 μl of 75% ethanol buffer with 1 μM ZnSO\(_4\). The digests were separated on a 5-μm Vydac 2.1 × 250-mm C18 HPLC column. Fractions were eluted with a 0–80% acetonitrile gradient over a period of 60 min. The fractions were collected and subjected individually to matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MALDI-R, Micromass, Manchester, UK) as described previously (23) with the only notable difference being that α-cyano-4-hydroxycinnamic acid dissolved in 50% methanol, 50% acetonitrile, 0.1% trifluoroacetic acid (Sigma) served as the matrix.

**Time of Flight**—Time of flight was measured using the following parameters: 3,300-V pulse voltage, 15,000-V source voltage, and 1,850 multichannel plate voltage. For each spectrum, 100 laser shots were averaged. The spectra were calibrated externally with yeast alcohol dehydrogenase tryptic peptides.

**To determine the minimum structure of PGN hydrolyzed by PGRP-L, the following synthetic PGN fragments were used:** muramyl dipeptide (MurNac-t-Ala-d-isoglutamin, >98% pure by HPLC and mass spectrometry, from Sigma), GlcNAc-MurNac-t-Ala-d-isoglutamin (>97% pure by HPLC and mass spectrometry, from Calbiochem, La Jolla, CA), MurNac-t-Ala-d-isoglutamin-L-Lys, MurNac-t-Ala-d-isoglutamin-L-Lys-β-Ala, and (GlcNAc-MurNac-t-Ala-d-isoglutamin-L-Lys-β-Ala), the latter three were synthesized as described previously (24–26, 58) and were >95% pure by silica-gel TLC (yielded one spot, using CHCl\(_3\)-methanol:acetate:hexafluoro-2-propanol = 1:3:0.1:0.1), mass spectrometry, and NMR. Five micrograms of each compound was incubated with 10 ng of PGRP-L or in buffer alone in 20 μl as described above for 48 h, and the digestion products were directly analyzed by mass spectrometry as described above.

**Bacteriolytic activity toward Micrococcus luteus** (ATCC 4868), *S. aureus* (strain Rb), and *Bacillus subtilis* (ATCC 6633) was measured spectrophotometrically as described previously (15) with 1.5 μg of PGRP-L and 500 μg of bacteria per milliliter. Lysozyme and lysisosphenin were used as positive controls and buffer alone as a negative control.

**Immunofluorescence**—COS-7 cells or HepG2/C3A (ATCC CRL-10741) human hepatoblastoma cells were grown on coverslips and transiently transfected with human PGRP-L in a pcDNA3 vector as described previously (14). For IF staining, the cells were washed with Dulbecco’s PBS without Ca\(^{2+}\) and Mg\(^{2+}\), fixed with 2% paraformaldehyde (no permeabilized cells for cell surface staining) or with 2% paraformaldehyde with 0.1% Triton X-100 (permeabilized cells for intracellular staining) at 4 °C for 30 min, washed with PBS, stained with primary anti-V5 mouse monoclonal antibodies (5 μg/ml, from Invitrogen, Carlsbad, CA), washed with PBS, stained with secondary goat-anti-mouse IgG-fluorescein isothiocyanate antibodies (Sigma), and observed in an IF microscope. The negative controls were cells transfected with an empty vector and stained as above, or cells transfected with PGRP-L as above and stained with the secondary antibody only (no V5

### Table I

| Mutant       | Primer sequence          |
|--------------|--------------------------|
| Human PGRP-L |                           |
| Δ1–343       | CACCATGCAGAGCTGAGCCAGCTC |
| Δ344–576     | CACCATGGCCAGTGGTCTCGAGC |
| Δ349–576     | CACCATGGCCAGTGGTCTCGAGC |
| H411A        | TGTCAGTGTGAATCACTCATGCTC |
| H436A        | ATGACGCGCTCAcGACAGACAGCA |
| W442A        | CACACGAGTGCcGcGACACAGCA |
| Y447A        | GAGACATCGGGcCAGTGGTGTGG |
| C530S        | TGTCGACCGGACcGCGGGGACCG |
| Human PGRP-S |                           |
| S178C        | CAGCCCTACCTGTCAGAGCCACCA |
| Mouse PGRP-S |                           |
| S165C        | CAAACGACTCTGTCAGGTCACCA |
| pcDNA3.1     |                           |
| Trans Bst11071/SspBI | CTATCGTGTGATCACTCATGCTC |
| Trans Bst11071/SspBI | CTATCGTGTGATCACTCATGCTC |
| Trans Bst11071/SspBI | CTATCGTGTGATCACTCATGCTC |

* Base substitutions are in lowercase.
**RESULTS**

**Human PGRP-L Is a Zn\(^{2+}\)-dependent N-Acetylmuramoyl-L-alanine Amidase**—To test if human PGRP proteins have PGN-lytic activity, we assayed whether soluble polymeric S. aureus PGN, labeled with biotin on the N-terminal glycan, was degraded by recombinant human PGRP-L, PGRP-I\(_\alpha\), PGRP-I\(\beta\), and PGRP-S. Only PGRP-L had PGN-lytic activity, similar to lysozyme and lysostaphin (positive controls), whereas PGRP-I\(\alpha\), PGRP-I\(\beta\), and PGRP-S did not, similar to buffer alone or trypsin (negative controls) (Fig. IA). The kinetics of PGN-lytic activity of PGRP-L (Fig. IB) was similar to the kinetics of PGN-lytic activity of lysozyme, and ~2–3 times slower than lysozyme (not shown).

Because PGRP-L has sequence homology to bacteriophage antibody. Both negative controls showed similar low level background fluorescence.

**Sequence Analysis**—DNA sequencing and alignments were done as described previously (14).

**Table II**

| Peptide                  | Theoretical mass | Observed mass |
|--------------------------|------------------|---------------|
| AEKAA + G2               | 603.31           | 604.15        |
| AEKAA + G4               | 646.31           | 646.22        |
| AEKAA + G5               | 703.33           | 703.527       |
| AEKAA + G5               | 774.37           | 774.32        |
| AEKAA + G6               | 831.39           | 831.48        |

T7 lysozyme, which is a Zn\(^{2+}\)-dependent PGN-lytic amidase (18), and because all four amino acids (His-411, Tyr-447, His-522, and Cys-530) corresponding to the Zn\(^{2+}\)-binding amino acids (His-17, Tyr-46, His-122, and Cys-130) in T7 lysozyme are conserved in PGRP-L, we next tested whether Zn\(^{2+}\) was also required for the PGN-lytic activity of PGRP-L. Chelating divalent cations with EDTA completely inhibited PGN-lytic activity of PGRP-L (Fig. 1C). This activity was restored by 1 mM (or 0.1 mM, not shown) Zn\(^{2+}\) and was only partially restored by 10 mM Mg\(^{2+}\) (Fig. 1C). These results demonstrate that Zn\(^{2+}\) is required for the PGN-lytic activity of PGRP-L.

The assay used in Fig. 1 detects conversion of PGN-bound biotin into low molecular weight fragments that migrate off the gel with the buffer front. Therefore, it measures degradation of PGN by several PGN-lytic enzymes of different specificities, including vertebrate lysozyme (which is a muramidase that digests PGN into disaccharide-peptides) and lysostaphin (which is an endopeptidase that digests a poly-glycine inter-peptide bridge of S. aureus PGN).

To determine which bond in PGN is hydrolyzed by PGRP-L, we next analyzed the products of digestion of soluble uncross-linked polymeric S. aureus PGN by PGRP-L using MALDI-TOF mass spectrometry. The peptides detected in PGN digests corresponded to the predicted (21, 27, 28) S. aureus PGN stem peptide (L-Ala-d-Glu-L-Lys-d-Ala-d-Ala) with one or two C-terminal d-Ala and with a side chain of two to six N-terminal Gly bound to l-Lys (Table II and Fig. 2A). These results indicate that human PGRP-L is an N-acetylglamoyl-L-alanine amidas (EC 3.5.1.28), which hydrolyzes the amide bond between the MurNac and l-Ala of PGN (Fig. 2A).

To determine the minimal structure of PGN hydrolyzed by PGRP-L, we next tested whether synthetic PGN fragments with peptide or glycan chains of various sizes were hydrolyzed by PGRP-L. The hydrolysis was measured by analyzing the digestion products using MALDI-TOF mass spectrometry. Muromyl dipetide (MurNac-l-Ala-d-isogln) and GlcNac-MurNac-l-Ala-d-isogln were not hydrolyzed by PGRP-L, whereas MurNac-l-Ala-d-isogln-L-Lys-d-Ala, and (GlcNac-MurNac-l-Ala-d-isogln-L-Lys-d-Ala)\(_2\) were all hydrolyzed by PGRP-L and yielded the expected peptides as digestion products (Fig. 2, B-D). These results further confirm the N-acetylmuramoyl-l-alanine amidase activity of human PGRP-L and demonstrate that MurNac-tripeptide is the minimal structure of PGN hydrolyzed by PGRP-L (Fig. 2B).

Incubations of human PGRP-L with intact bacteria (M. luteus, S. aureus, and B. subtilis) for up to 3 days at 37 °C did not reduce the optical density of bacterial suspensions (not shown). These results indicate that human PGRP-L is not bacteriolytic for intact bacteria, which is similar to insect PGRP-SC1b (12), but contrasts with other PGN-lytic enzymes, such as lysozyme or lysostaphin, which are bacteriolytic and were used here as positive controls. Thus, although all these bacteria contain
FIG. 2. PGRP-L is an N-acetylmuramoyl-L-alanine amidase that digests polymeric PGN and synthetic MurNAc-tripeptide. The cleaved bond in PGN (A) and its minimal fragment hydrolyzed by PGRP-L, MurNAc-tripeptide (B) were determined by identifying peptides (using MALDI-TOF MS) released from polymeric uncross-linked PGN (Table II) and synthetic MurNAc-tripeptide (C) and MurNAc-tetrapeptide (D) after incubation with PGRP-L (lower panels in C and D) but not after incubation in buffer alone (upper panels in C and D). The observed masses of peptides obtained from digestion of MurNAc-tripeptide (C) and MurNAc-tetrapeptide (D) are shown on the figure, and their theoretical masses are: Ala-isoGln-Lys, 346.1 [M + H]+ and 368.1 [M + Na]+; Ala-isoGln-Lys-Ala, 417.2 [M + H]+ and 439.2 [M + Na]+. The vertical scale in the upper panels (in C and D) was expanded to reveal minor peaks. The results are from one of two similar experiments. Digestion of (GlcNAc-MurNAc-L-Ala-D-isoGln-L-Lys-D-Ala)_2 yielded the same peptides (not shown), whereas MurNAc-L-Ala-D-isoGln and GlcNAc-MurNAc-L-Ala-D-isoGln were not hydrolyzed by PGRP-L (not shown).
Peptidoglycan Recognition Protein-L Is an Amidase

PGN that is potentially susceptible to amidase digestion, PGN in intact bacteria is not susceptible to or not available for digestion by insect and human PGRP amidases.

**Amino Acid Sequences Needed for Amidase Activity of PGRP-L**—To determine which amino acid sequences are required for the amidase activity of human PGRP-L (and possibly other PGRPs), we compared the amino acid sequence of bacteriophage T7 lysozyme with the sequences of mammalian and insect PGRPs, including the amidase-active D. melanogaster PGRP-SC1b (12) (Fig. 3). The sequences of both mammalian and insect short PGRPs and the C-terminal regions of long PGRPs are highly conserved and are homologous to the bacteriophage T7 lysozyme, which is a member of a family of bacteriophage and bacterial type 2 N-acetylmuramoyl-L-alanine amidases (Fig. 3). This homologous sequence is referred to as an amidase type 2 domain or PGRP domain and is subdivided into PGRP domains I, II, and III (Fig. 3) (14). Based on this homology, the amidase domain of PGRP-L (containing PGRP domains I, II, and III) should be required and sufficient for the amidase activity of PGRP-L. To test this hypothesis, we generated two PGRP deletion mutants and tested their PGN-lytic activity:

- **/H9004 1–343** PGRP-L deletion mutant had PGN-lytic activity, although lower than the full-length PGRP-L, whereas **/H9004 344–576** PGRP-L deletion mutant had no PGN-lytic activity (Fig. 4B). These results demonstrate that the C-terminal region of PGRP-L containing all three PGRP domains (approximately 40% of the full-length sequence) is required and sufficient for the amidase activity of PGRP-L, although its activity is reduced compared with the full-length molecule. The N-terminal region of PGRP-L (approximately 60% of the full-length sequence) is not absolutely necessary for amidase activity. **Fig. 3.** Alignment of C-terminal amino acid sequences of mammalian and insect PGRPs with T7 lysozyme: conserved amino acids required for Zn²⁺ binding and amidase activity. Identities are shaded darker and similarities are shaded lighter. PGRP domains I, II, and III (underlined) and conserved cysteines (boxed) are boxed. The amidase-active D. melanogaster PGRP-SC1b (12) (Fig. 3) is boxed. The mutated amino acids in PGRP-L (used in Fig. 4) are marked by arrows. Abbreviations and GenBank™ or SwissProt accession numbers are as follows. Mammals: *Bt*, *Bos taurus* (cow; PGRP-S, AY083309); *Cd*, *Camelus dromedarius* (camel; PGRP-S, AJ131676); *Hs*, *Homo sapiens* (human; PGRP-L, AF384856; PGRP-Ia, AY083576; PGRP-Ib, AY083577; PGRP-S, AF074833; Mm, *Mus musculus* (mouse; PGRP-L, AF149837; PGRP-S, AF074832; Rn, *Rattus norvegicus* (rat; PGRP-S, AF154114). Insects: *Ag*, *Aedes aegypti* (mosquito; PGRP-L, AF074831; EAA08262; PGRP-S2, EAA08262; PGRP-S3, EAA08263; PGRP-LA1, EAA08264; PGRP-LA2, EAA08265; PGRP-LB, EAA08266; PGRP-LC, EAA08267).
required for the amidase activity of PGRP-L, although it contributes to the full enzymatic activity of PGRP-L.

To determine which sequences in the C-terminal region of PGRP-L are needed for the amidase activity, we deleted PGRP domain I (Δ495–576) or created single amino acid substitutions (Figs. 3 and 4). Previous extensive mutational and crystallographic analysis of T7 lysozyme (18) revealed that His-17, Tyr-46, His-122, and Cys-130 are required for both Zn\(^{2+}\) binding and amidase activity of T7, and at least three other amino acids (His-36, Trp-41, and Lys-128) are also required for the amidase activity of T7 (Fig. 3), although some mutations in the latter 3 positions reduce, but do not totally abolish its amidase activity. Also, D. melanogaster PGRP-SC1b has threonine corresponding to Lys-128 in T7 lysozyme, showing no absolute requirement for Lys-128 for the amidase activity (12). To test the requirement for these amino acids for the amidase activity of PGRP-L, we created several single alanine substitution mutants targeting the homologous amino acids in human PGRP-L (Fig. 3). We constructed a C530S mutant, because all other amidase-inactive PGRPs have serine in this position (Fig. 3) and because the corresponding C168S mutation in D. melanogaster PGRP-SC1b abolished its amidase activity (12). We also created a C419A mutation, because a mutation in the corresponding cysteine (Cys-80) in D. melanogaster PGRP-SA abolished its Toll receptor-activating capacity (6).

The Δ495–576 PGRP-L deletion mutant, as well as Y447A and C530S amino acid substitutions, targeting the PGRP-L amino acids homologous to the amino acids needed for Zn\(^{2+}\) binding in T7 lysozyme, and also C419A mutation, totally abolished PGN-lytic activity of PGRP-L (Fig. 4B). H411A and H436A mutants had full amidase activity, and W442A mutant had reduced amidase activity (Fig. 4B). These results show that all three PGRP domains, as well as Tyr-447 and Cys-530 (corresponding to the Zn\(^{2+}\)-binding Tyr-46 and Cys-130 in T7 lysozyme) are required for the amidase activity of PGRP-L. They also show that, in contrast to T7 lysozyme, His-411 and His-436 (corresponding to His-17 and His-36 in T7 lysozyme) are not required for the amidase activity of PGRP-L, perhaps because in PGRP-L in the absence of His-411 and His-436, the adjacent His-410 and Tyr-435 could assume the Zn\(^{2+}\) binding capacity. Of note, all mammalian and insect PGRPs with predicted amidase activity (see below) have His in both positions corresponding to His-410 and His-411 in human PGRP-L (Fig. 3). Cys-419 and Cys-425 are conserved in 94% of all insect and mammalian PGRPs and are likely involved in a disulfide bond that is required for the proper conformation of both amidase-active and amidase-inactive PGRPs (6, 17). However, these two Cys are not conserved in the T7 lysozyme. Therefore, these results suggest conservation between mammalian and insect PGRPs and bacteriophage T7 lysozyme of the Zn\(^{2+}\)-binding amino acids and partial conservation of other amino acids needed for the amidase activity.

Both human and mouse PGRP-S do not have amidase activity, although they have seven out of eight of the above amino acids, needed for the amidase activity of human PGRP-L, D. melanogaster PGRP-SC1b, or T7 lysozyme (Fig. 3). The eighth amino acid is serine, in the position homologous to Cys-530 in human PGRP-L, Cys-168 in D. melanogaster PGRP-SC1b, and Cys-130 in T7 lysozyme (Fig. 3). To test if these eight amino acids were sufficient for the amidase activity, we constructed S178C and S165C mutations in human and mouse PGRP-S, respectively, and tested their amidase activity. However, neither of these mutants had amidase activity (Fig. 4B), which shows that, although these eight amino acids are required for the amidase activity, they are not sufficient. Thus, there are additional amino acids that are required for the amidase activity of PGRPs.

Location of PGRP-L in Cells—Human PGRP-L sequence has two predicted transmembrane domains and, therefore, PGRP-L is predicted to be a transmembrane protein, located in the cell membrane with the N and C termini outside the cell (14). However, a 64-kDa N-acetylmuramoyl-L-alanine amidase with the 15-amino acid N-terminal sequence (SLPLLMDSVIQALAE) identical to human PGRP-L (14) was previously...
Peptidoglycan Recognition Protein-L Is an Amidase

Our results identify the function of human PGRP-L as an N-acetylmuramyl-L-alanine amidase (EC 3.5.1.28), an enzyme that hydrolyzes the amide bond between the MurNac and L-Ala of bacterial PGN. The minimum PGN fragment hydrolyzed by PGRP-L is MurNac-tripeptide. Our results also show that the other members of human PGRP family, PGRP-Ia, PGRP-Ib, and PGRP-S, do not have PGN-lytic activity. The presence of N-acetylmuramyl-L-alanine amidase was reported before in human and animal serum and tissues (29–36), but the cDNA of the enzyme had not been cloned and its gene had been unknown. Therefore, our results are the first to identify the cDNA and the gene for a mammalian (human) amidase (located on chromosome 19) (14).

PGN is a unique and essential component of the cell wall of virtually all bacteria and is not present in eukaryotes (21, 27, 28), and thus PGN is an excellent target for the innate immune system (20). Indeed, higher eukaryotes, including mammals, have several PGN recognition molecules (20). They include three pattern recognition receptors, which are members of the leucine-rich repeat protein family: cell-surface CD14 and Toll-like receptor 2, and intracellular nucleotide-binding oligomerization domain (Nod)-containing proteins, Nod1 and Nod2 (20). CD14 and Toll-like receptor 2 recognize extracellular polymeric PGN, and Nod1 and Nod2 recognize intracellular PGN fragments (20). Nod2 recognizes muramyl dipeptide and other muramyl peptides (25, 37), and Nod1 recognizes diaminopimelic acid-containing peptides from PGN from Gram-negative bacteria and Gram-positive bacilli (26, 38). Interaction of PGN with these receptors has highly pro-inflammatory effects due to the induction of secretion of numerous chemokines and cytokines and other mediators of inflammation (20, 28, 39). The combined effect of these mediators in vivo is responsible for the normal antimicrobial host defenses and can be observed as typical clinical manifestations of bacterial infections, such as inflammation, pus formation, leukocytosis, fever, acute-phase response, hypotension, sleepiness, decreased appetite, and arthritis (28, 39, 40). Microbial stimulation of the innate immune system is also required for the initiation of the adaptive immune response, which accounts for the well-known immune adjuvant effect of microbial components, including PGN (41–44).

Animals also have many bacterial recognition proteins that play a scavenger function, i.e. they remove, kill, digest, and detoxify bacteria and their pro-inflammatory components (20, 45, 46). They include phagocytic (e.g. scavenger) receptors on phagocytic cells and bacteriolytic enzymes that digest bacteria in phagolysosomes. Serum, body secretions, and phagocytic cell granules contain abundant amounts of lysozyme, an enzyme that hydrolyzes the glycosidic bond between MurNac and GlcNac of PGN (47, 48). Lysozyme has direct bactericidal activity only against few bacteria, and its main function is to digest PGN (46–49). Complete lysozyme digestion of PGN produces disaccharide-peptides (from uncross-linked PGN) or peptide-cross-linked disaccharides (from peptide-cross-linked PGN). Although these PGN fragments have often reduced proinflammatory activity (50, 51), they are still biologically active (52) and can induce proinflammatory responses in host cells, e.g. through the recently identified Nod1 and Nod2 proteins (25, 26, 37, 38).

The N-acetylmuramyl-L-alanine amidase removes stem peptides from the PGN molecule, which further reduces or eliminates the biologic activity of PGN. Indeed, digestion of PGN with insect PGRP-SC1b, which is an amidase, abolishes its cell-activating capacity (12), and digestion of PGN with human serum amidase also reduces or abolishes the pro-inflammatory activity of PGN (53). Thus, due to its amidase activity, human PGRP-L, similar to insect PGRP-SC1b (12), is likely to play a scavenger role by digesting biologically active PGN into biologically inactive fragments. PGRP-L has no direct bacteriolytic activity, similar to the serum amidase and D. melanogaster PGRP-SC1b, and probably functions in conjunction with lysozyme (12, 32, 34, 53) and other bactericidal and bacteriolytic host defense mechanisms.

PGRP-L is likely to be the only human N-acetylmuramyl-L-alanine amidase and is also likely to be the product of the same gene as the previously reported amidase found in human serum (29–36), because: (a) the remaining three human PGRPs do not have an amidase activity; (b) the 15-amino acid N-terminal sequence of the serum amidase (29) is identical to the predicted N-terminal sequence of mature PGRP-L after cleavage of the signal peptide (14); (c) serum amidase and PGRP-L have a similar molecular mass of ~65 kDa (glycosylated) and ~60 kDa (deglycosylated) (14, 29, 36); (d) serum amidase and PGRP-L cleave the same bond (29–36); (e) both serum amidase and PGRP-L have the same specificity.

**Fig. 5.** Expression of recombinant PGRP-L in cells. COS-7 cells were transiently transfected with PGRP-L (top panels) and the expression of PGRP-L protein on the cell surface in nonpermeabilized cells (left) or in permeabilized cells (intracellular expression, right) was determined by IF with anti-V5 antibodies (magnification, 10 × 40). Nonpermeabilized and permeabilized cells transfected with an empty vector (no PGRP-L control) are shown in the lower panels. The results are from one of three similar experiments.
for MurNAc-tripeptide and do not hydrolyze muramyl-dipeptide and GlcNAc-MurNAc-dipeptide (35); (f) PGRP-L mRNA is highly abundant in the liver, which makes several serum proteins, and amidase is present in human serum at a high concentration (100 µg/ml) (29); and (g) no other human genes with PGRP/amidase domain could be found (13, 14). However, serum amidase is a secreted protein, whereas PGRP-L has two predicted transmembrane domains, and in transiently transfected cells PGRP-L is not secreted but localizes primarily to intracellular vesicles and to a lower extent to plasma membrane. The PGRP-L-containing vesicles may be associated with Golgi and may be destined either for secretion or membrane expression. However, the processing of the recombinant protein may be different in transfected cells in vitro than of the native protein in vivo, and the latter will have to be studied (once the reagents to detect native PGRP-L protein in vivo become available).

The tissue source of the serum amidase is not known, but in addition to its presence in serum, earlier studies also detected amidase activity or immunoreactive protein (similar to the serum amidase) in various cells and tissues, including intestinal mucosa (but not intestinal lumen) (34), liver, spleen, arthritic synovial tissue, lymph nodes, and bone marrow and blood neutrophils and eosinophils (36). Human PGRP-L is highly expressed in the liver and has lower expression in other tissues, such as parts of the intestinal tract, lymph nodes, thymus, heart, pancreas, and bone marrow (14), which is consistent with the expression of the serum amidase, except for its presence in neutrophils and eosinophils (36). The amidase immunoreactivity in neutrophils and eosinophils (36), however, could have been due to the cross-reactivity of the antiserum with PGRP-S. PGRP-S is highly expressed in these cells and was not known at the time of the study.

Therefore, PGRP-L and serum amidase are either the same protein, or a product of the same gene, but alternatively spliced or differentially processed. The existence of multiple splice forms of human PGRP-L is likely, because we have detected at least three different mRNA transcripts of human PGRP-L (14) and because during cloning of human PGRP-L cDNA we have noticed additional PGRP-L splice forms (14). The existence of five alternative splice forms of mouse PGRP-L has been reported (54, 55), and both Drosophila and Anopheles PGRPs have several splice forms (1–3).

Whereas in mammals only one PGRP is an amidase, in insects several PGRPs are likely to be amidases. Amidase activity of Drosophila PGRP-SC1b has been demonstrated (12), and, based on five amino acids conserved (Figs. 3) and do not have PGN amidase activity (configuration, yet this PGRP-S mutants still do not have any amidase activity (Fig. 4). Moreover, human and mouse PGRP-S also have three additional amino acids conserved, corresponding to Cys-419 in human PGRP-L and His-36 and Trp-41 in T7 lysozyme (that are needed for their amidase activity) (Fig. 3). Therefore, additional, so far unidentified amino acids, not conserved between PGRP-L and PGRP-S (which have overall 42% identity and 54% similarity), are required for the amidase activity of mammalian PGRPs.

In conclusion, our results indicate that one function of PGRPs, i.e. N-acetylglucosaminyl-l-alanine amidase activity, is shared by one mammalian and at least one (or likely more) insect PGRP. Functions of several other PGRPs are different in mammals and insects, and functions of two mammalian and several insect PGRPs are still not known (20). After this report was completed, the PGRn-lytic activity of Drosophila PGRP-LB (56) and N-acetylglucosaminyl-l-alanine amidase activity of mouse PGRP-L were reported (57).

Acknowledgments—We are grateful to Hakan Steiner for communicating to us his results on the amidase activity of Drosophila and mouse PGRPs.

REFERENCES

1. Werner, T., Liu, G., Kang, D., Ekengren, S., Steiner, H., and Hultmark, D. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 13752–13757

2. Christopoulos, G. K., Zhdanov, E., Barillas-Mury, C., Birney, E., Blandin, S., Blais, C., Brey, P. T., Collina, F. H., Danielli, A., Dimopoulos, G., Hertsu, C., Hoa, N. T., Hoffmann, J. A., Kanoko, S. M., Letunie, I., Levashina, E. A., Lekker, T. G., Lygett, G., Meers, S., Michel, K., Moita, L.F., Muller, H.-M., Oota, M., Paasikwitz, S. M., Reichhart, J. M., Zbetski, A., Tren, L., Venn, K. D., Vlahou, D., Volz, J. von, Murer, C., Xu, J. Zheng, L., Bork, P., and Kafatos, F. C. (2002) Science 295, 158–165

3. Werner, T., Berg, Reenber, K., Melotto, P., Steiner, H., and Hultmark, D. (2003) J. Biol. Chem. 278, 26319–26322

4. Yoshida, H., Kinoshita, K., and Ashida, M. (1996) J. Biol. Chem. 271, 12854–12860

5. Takehana, A., Katsuyama, T., Yano, T., Oshima, Y., Takada, H., Aigaki T., and Kurata, S. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 13750–13710

6. Michel, T., Reichhart, J.-M., Hoffmann, J. A., and Royet, J. (2001) Nature 414, 756–759

7. Hoffmann, J. A., and Reichhart, J.-M. (2002) Nat. Immunol. 3, 121–126

8. Leudl, P., Parquet, C., Fili-Flourney, S., Raya, J.-H., Corbeil, M., Lee, W.-J., Megan-Lecreulx, and D., Lamaitre, B. (2003) Nature Immunol. 4, 478–484

9. Choe, K.-M., Werner, T., Stoven, S., Hultmark, D., and Anderson, K. V. (2002) Science 296, 359–362

10. Guttman, M., Gohert, V., Michel, T., Belvin, M., Doyk, G., and Hoffmann, J. A. (2002) Nature 416, 640–644

11. Ramet, M., Manfreuli, P., Pearson, A., Mathey-Prevot, B., and Eeskowitz, R. A. (2002) Nature 416, 644–648

12. Melotto, P., Karlsson, J., and Steiner, H. (2003) J. Biol. Chem. 278, 7059–7064

13. Kang, D., Liu, G., Lundstram, A., Gellius, E., and Steiner, H. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 10078–10082

14. Liu, C., Xu, Z., Gupta, D., and Dziarski, R. (2001) J. Biol. Chem. 276, 34586–34594

15. Liu, C., Gellius, E., Liu, G., Steiner, H., and Dziarski, R. (2000) J. Biol. Chem. 275, 24419–24499

16. Dziarski, R., Platt, K. A., Gesch, E., and Steiner, H. (2003) J. Biol. Chem. 278, 10689–10697

17. Tylde, C. K., Yount, N., Tran, D., Yuan, J., and Selsted, M. E. (2002) J. Biol. Chem. 277, 16958–16964

18. Cheng, X. Zhang, X., Xing, J., and Studier, E. W. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4034–4038

19. Dunn, J., and Studier, P. W. (1983) J. Biol. Chem. 258, 477–535

20. Dziarski, R. (2003) Cell. Mol. Life Sci. 60, 1783–1804

21. Roseenthal, R. S., and Dziarski, R. (1994) Methods Enzymol. 235, 253–285

22. Dziarski, R., Tapping, R. I., and Tobias, P. S. (1998) J. Biol. Chem. 273, 8680–8690

23. Xu, N., Huang, Z.-H., de Jonge, B. L. M., and Gage, D. A. (1997) Anal. Biochem. 248, 7–14

24. Kusumoto, S., Tanuma, Y., Inakuma, K., and Shiba, T. (1976) Bull. Chem. Soc. Jpn. 49, 533–539

25. Inohara, N., Ogura, Y., Fontalba, A., Gutierrez, O., Pons, F., Crespo, J., Fukase, K., Inamura, S., Kusumoto, S., Hashimoto, M., Foster, S. J., Moran, A. P., Fernandez-Luna, J. L., and Nunez, G. (2003) J. Immunol. 166, 5512–5518

26. Chamaillard, M., Hashimoto, M., Horie, Y., Masumoto, J., Qiu, S., Saab, L., Ogura, Y., Kawasaki, A., Fukase, K., Kusumoto, S., Valvano, M. A., Foster, S. J., Sakabe, T., Wang, N., Nunez, G., and Inohara, N. (2005) Nature Immunol. 7, 702–707

27. Schleifer, K. H., and Kandler, O. (1972) Bacteriol. Rev. 36, 407–477

---

a R. Dziarski, unpublished data.
Human Peptidoglycan Recognition Protein-L Is an N-Acetylmuramoyl-L-alanine Amidase
Zheng-Ming Wang, Xinna Li, Ross R. Cocklin, Minhui Wang, Mu Wang, Koichi Fukase, Seiichi Inamura, Shoichi Kusumoto, Dipika Gupta and Roman Dziarski

J. Biol. Chem. 2003, 278:49044-49052.
doi: 10.1074/jbc.M307758200 originally published online September 23, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M307758200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 50 references, 24 of which can be accessed free at
http://www.jbc.org/content/278/49/49044.full.html#ref-list-1