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A Novel Antimicrobial Peptidoglycan Recognition Protein in the Cornea

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Abstract

Purpose—In an earlier gene expression study, the authors identified a novel antimicrobial gene, Peptidoglycan recognition protein 1 (*Pglyrp1*), in the mouse cornea. Here the expression of the *Pglyrp1* transcript and the encoded protein, PGLYRP1, in the cornea was investigated. The role of PGLYRP1 in the cornea was further investigated using wild-type and *Pglyrp1*-deficient mice. This is the first report of this antimicrobial protein in the cornea.

Methods—PGLYRP1 was detected in the cornea and was further localized to the epithelium by immunohistology, confocal microscopy, immunoblotting, and real-time PCR. The role of PGLYRP1 in the cornea was investigated by comparing the response of wild-type and *Pglyrp1*^{−/−} mice to corneal epithelial wounds and *Pseudomonas aeruginosa*-mediated corneal infections. The antibacterial effects of corneal PGLYRP1 were assayed by measuring bacterial growth in vitro, in the presence of wild-type corneal epithelial extracts, before and after antibody-mediated blocking of PGLYRP1.

Results—PGLYRP1 is expressed at high levels in the mouse corneal epithelium. PGLYRP1 was localized to the mouse corneal epithelium and the human corneal epithelium. The *Pglyrp1*^{−/−} mouse shows delayed healing and poor clearing of bacterial keratitis; in vitro its epithelial protein extract shows reduced bacteriostatic activity compared with wild-type mice.

Conclusions—PGLYRP1 is a novel antimicrobial protein of the corneal epithelium and protects the ocular surface from bacterial infections.

In a genomewide differential gene expression study of the mouse cornea, lens, and tendon, we identified the expression of a gene, *Pglyrp1*, encoding a novel antimicrobial protein, PGLYRP1, in the cornea.¹ Here we further investigated this antimicrobial protein in the cornea. The peptidoglycan recognition proteins (PGRPs) are a large family of pathogen-associated pattern recognition molecules in insects, mollusks, echinoderm, and vertebrates.^{2,3} Mammalian PGRPs, a family of four distinctive homologous gene products, were identified recently. These were originally named peptidoglycan recognition protein-short (*PgrpS*), peptidoglycan recognition protein-long (*PgrpL*), peptidoglycan recognition protein-intermediate alpha and beta (*Pgrp Ia* and *Pgrp Ib*). These have been now renamed *Pglyrp1*,

Pglyrp2, *Pglyrp3*, and *Pglyrp4* in the mouse, based on the names given to the corresponding human homologues by the HUGO Gene Nomenclature Committee.^{4,5} Accordingly, peptidoglycan recognition protein short (PGRPS), the subject of the present study, will be referred to as PGLYRP1, and the mouse gene will be referred to as *Pglyrp1*. The PGLYRP1 protein has a restricted distribution in the bone marrow and tertiary granules of mature neutrophils.⁴ PGLYRP2, a widely expressed, secreted membrane protein, is present in the liver, serum, colon, lymph nodes, heart, thymus, and pancreas. PGLYRP3 and PGLYRP4 are also widely expressed in the esophagus, thymus, skin, and other mucous membranes.⁶ PGLYRP2 is a Zn²⁺-dependent amidase, hydrolyzes peptidoglycans from bacterial cell walls, and may be important in reducing levels of proinflammatory peptidoglycans to dampen overstimulation of the immune system.⁶ PGLYRP1, PGLYRP3, and PGLYRP4 interact with bacterial peptidoglycans, interfere with the biosynthesis of peptidoglycans, and are bactericidal or prevent further growth of bacteria.⁶ The concept that peptidoglycan recognition proteins have a role at the ocular surface is very new.

The cornea is the outermost barrier tissue of the eye that is often under attack by fungal, bacterial, and viral pathogens.^{7,8} Tear film- and membrane-associated mucins provide the first barrier to microorganisms.⁹ Stratified epithelium and shedding of the surface cells provide a second level of protection for the corneal surface.¹⁰ The cornea has an active innate immune signaling mechanism through the toll-like receptors (TLR).^{11,12} Even lumican, a resident stromal structural protein, contributes to TLR-mediated innate immune response of the cornea.¹³ Another major defense mechanism at the ocular surface involves antimicrobial proteins and peptides. Antimicrobial peptides identified in the cornea thus far include the defensins,¹⁴ antifungal histatins,⁸ and cathelicidin.¹⁵ Several chemokines in the cornea, such as, MIP-3 α and thymosin β 4, have antimicrobial properties in vitro and in vivo.⁸ Of the PGLYRP family of antimicrobial proteins, there has been only one report of PGLYRP2 expressed at low levels in the corneal epithelium and upregulated in corneal epithelial cell cultures after exposure to bacteria.¹⁶

In the present study, we show that *Pglyrp1* is constitutively expressed in mouse corneal epithelial cells. At the protein level, it is detectable in the mouse and human corneal epithelium. To date this particular member, PGLYRP1, has been detected in bone marrow and circulating neutrophils.⁴ Our finding of PGLYRP1 in the corneal epithelium suggests a novel role for this protein in the cornea, implicating another level of antimicrobial defense at the ocular surface. With the use of mice deficient in the *Pglyrp1* gene, we investigated how PGLYRP1 functions in the cornea and how its deficiency affects the cornea.

Materials and Methods

Animals

BALB/c and *Pglyrp1*^{-/-} mice⁴ backcrossed to the BALB/c background for eight generations (provided by Roman Dziarski) were housed in a specific pathogen-free facility according to policies approved by the Animal Care Committee of Johns Hopkins University. All experiments using mice were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Immunoblotting

The corneal epithelium was separated as a sheet after intact corneas were incubated in 250 μ L of 20 mM EDTA (sterile, pH 7.4) for 30 minutes at 37°C. Total protein extracts in reagent (T-PER; Pierce Biotechnology, Rockford, IL) were prepared from whole corneas and the isolated corneal epithelium. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes with a transblot apparatus (Bio-Rad, Hercules, CA) at 100 V for 1

hour at 4°C. Nonspecific binding sites were blocked overnight with 3% gelatin (Sigma, St. Louis, MO) in Tris-buffered saline containing 0.1% Tween-20. Immunoblots were incubated with an anti-mouse PGLYRP1 antibody (AF 2696; R&D Systems, Inc., Minneapolis, MN) at a final dilution of 1:500. Membranes were washed and incubated with peroxidase-conjugated donkey anti-goat IgG (sc-2020; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Protein bands were detected with the enhanced chemiluminescence kit (GE Healthcare, Chalfont, St. Giles, UK). To confirm equal loading, actin was immunostained with an anti-actin polyclonal antibody (1:2000; sc-1615; Santa Cruz Biotechnology, Inc.).

Quantitative Real-Time PCR

Total RNA was extracted from isolated corneal epithelial sheets in 1 mL reagent (TRIzol; Invitrogen Corp., Carlsbad, CA).¹⁷ First-strand cDNA was synthesized from 1 µg total RNA with a reverse transcriptase enzyme (Superscript II; Invitrogen Corp.) and oligo (dT) primers. Resultant cDNA samples were stored at -20°C until use. Quantitative real-time PCR (qRT-PCR) was carried out with a sequence detection system (ABI PRISM 7900HT; Applied Biosystems, Foster City, CA) and PCR master mix (SYBR Green; Applied Biosystems). Reaction conditions were 50°C for 2 minutes, 95°C for 10 minutes, and 45 cycles of 95°C for 15 seconds and 59°C and 72°C for 30 seconds each. Specificity of the PCR products was confirmed by examination of the dissociation curves of the products. Primers used were as follows: *Gapdh*, (forward) 5'-CCCTGGCCAAGGTCATCC-3', (reverse) 5'-TGATGGCATGGACTGTGGTC-3'; *Pglyrp1*, (forward) 5'-GCAATGTGCAGCATTACCAC-3', (reverse) 5'-CTGTGTGGTCACCCTTGATG-3'. A threshold cycle number (C_T) was determined using the ABI-SDS software, and a mean C_T value was obtained from triplicate reactions. The threshold cycle difference was calculated as follows: $\Delta C_T = (C_T \text{ of } Pglyrp1 - C_T \text{ of } Gapdh)$, and relative expression was defined as $2^{-\Delta C_T}$.

Confocal Microscopy

Whole mouse eyes were frozen in OCT (Tissue-Tek; Sakura Finetek, Zoeterwoude, Netherlands), and 20-µm-thick sections were prepared.¹⁸ Cryosections of the human cornea were kindly provided by Albert Jun (Johns Hopkins University). Mouse and human cornea cryosections were methanol-fixed and blocked with 2% BSA in PBS containing 0.2% nonionic surfactant (Triton X100; Sigma) for 30 minutes at room temperature. The sections were washed thoroughly and incubated with primary antibodies against PGLYRP1 (mouse, AF 2696 [R&D Systems, Inc.] at 1:500 dilution; human, sc-55745 [Santa Cruz Biotechnology Inc.] at 1:100 dilution) at room temperature. A fluorescence-labeled secondary antibody (A-21446, Alexa Fluor 647 anti-goat IgG; Molecular Probes, Eugene, OR) was used at a 1:400 dilution for 30 minutes at room temperature. The sections were counterstained with 4',6'-diamidin-2-phenylindole (DAPI; 1 µg/mL). Negative controls consisted of sections treated as described but without the primary antibody. Imaging was performed under a single-photon microscope (LSM 510 Meta Confocal; Zeiss) using 20×, 40× or 100× oil immersion objectives.

Bacteriostatic Activity Assay

To test for bacteriostatic activity, corneal epithelial extracts from *Pglyrp1*^{+/+} and *Pglyrp1*^{-/-} mice were incubated at 37°C for 0, 60, and 120 minutes, with an early log-phase culture of *Escherichia coli* DH 5α in 96-well plates containing 1% LB broth in PBS. A maximum of 5 µg protein was used per well, maintaining a ratio of approximately 50 cfu/µg corneal epithelial extract. Anti-PGLYRP1 antibody, 1:40 dilution, was included with a set of *Pglyrp1*^{+/+} corneal extracts to test whether blocking its target, PGLYRP1, would reduce bacteriostatic activity of the wild-type extract. All conditions were tested in triplicate wells, and the data were presented as the average number of bacterial cfu/well ± SEM.

***Pseudomonas aeruginosa* Corneal Infection**

Pglyrp1^{+/+} and *Pglyrp1*^{-/-} mice were infected with *Pseudomonas aeruginosa* as described.¹⁹ The mice were anesthetized by intraperitoneal injection (0.014 mL/g body weight) of 12.5 mg/mL tribromoethanol. One cornea per animal ($n = 4$) was scarified with a 26-gauge needle, and 4.5×10^5 cfu bacteria in 2.5 μ L Dulbecco modified Eagle medium was introduced to the cornea. The eyes were examined under a dissecting microscope (SMZ 1500; Nikon, Tokyo, Japan) fitted with a fluorescein/FITC filter. The images were captured with a digital camera (DXM 1200; Nikon) at 0, 6, and 24 hours and 7 days after infection. In a second similar experiment, we used six mice per group. One cornea per animal was scarified, and 8×10^5 cfu bacteria were applied. Corneas were harvested 5 days after infection and homogenized, and up to three different dilutions were plated in nutrient agar plates, in triplicate, to obtain total bacterial counts.

Corneal Wounding

The mice were anesthetized, and one eye was treated topically with proparacaine hydrochloride 0.5% (Bausch and Lomb Pharmaceuticals, Inc., Tampa, FL) before wounding; the other eye was left uninjured. To study wound healing, circular wounds were made with an Alger brush II (0.5 mm; Katena Products, Inc., Denville, NJ) as described.²⁰ With the use of a Hamilton syringe, wounded corneas were inoculated with 1 μ L (10 μ g/ μ L) *Staphylococcus aureus* peptidoglycans (Sigma). Fluorescein sodium benoxinate hydrochloride ophthalmic solution (Bausch and Lomb Pharmaceuticals, Inc.) was used to visualize the wound margins. At 0 and 24 hours after wounding, the eyes were photographed as described. Captured images were analyzed with image analysis software (NIS-Elements AR 3.0; Nikon). Residual wound, expressed as a percentage of the original wound area, was taken as a measure of the extent of healing.

Results

Presence of PGLYRP-1 at High Levels in the Cornea

Our earlier microarray gene expression study indicated strong expression of *Pglyrp1* in the mouse cornea.¹ Here we tested whether *Pglyrp1* is expressed in the mouse corneal epithelium by qRT-PCR. *Pglyrp1* mRNA, compared with *Gapdh*, was measured in total RNA extracts of the corneal epithelium from wild-type mice. The results indicated strong expression of *Pglyrp1* in the epithelium, with relative expression approximately twofold higher than that of the housekeeping *Gapdh* gene (Table 1).

We next sought to determine the presence of the PGLYRP1 protein in the mouse cornea by immunoblotting. A single PGLYRP1 band of the expected size (approximately 16 kDa) was seen in protein extracts of the whole cornea (Fig. 1A). Total protein extract from the bone marrow, used as a positive control, yielded a PGLYRP1 protein band of the same size. The PGLYRP1-deficient mouse (*Pglyrp1*^{-/-}) bone marrow and corneal extracts were negative for PGLYRP1, as expected.

In previous studies, the PGLYRP1 protein had been detected in the bone marrow and circulating neutrophils only; therefore, we wanted to further confirm our finding of PGLYRP1 in the cornea. RT-PCR results indicated *Pglyrp1* transcript expression in the corneal epithelium. We next sought to determine the presence of PGLYRP1 in corneal epithelium by immunoblotting total protein extracted from the isolated epithelium (Fig. 1B). Taken together, these experiments suggest constitutive high expression of *Pglyrp1* in the corneal epithelium.

To further localize PGLYRP1 within the corneal epithelium, frozen sections of the cornea from wild-type (*Pglyrp1*^{+/+}) and *Pglyrp1*^{-/-} mice were immunostained with an anti-PGLYRP1 antibody, and the sections were viewed by confocal microscopy. PGLYRP1 was clearly visible in the differentiated superficial and the upper wing cells of the epithelium from *Pglyrp1*^{+/+} mouse corneas. Furthermore, it was not detectable in the basal undifferentiated cell layer of the cornea. As expected, the *Pglyrp1*^{-/-} mouse corneas were negative for PGLYRP1 immunostaining (Fig. 2). Frozen sections of human cornea were immunostained to test for the presence of PGLYRP1. As in the mouse, PGLYRP1 was detected primarily in the superficial layers of the epithelium (Figs. 3B, D, J, L). The results also indicated that stroma in the mouse and human corneas were negative for PGLYRP1 (Figs. 2B, 3B).

Reduced Antimicrobial Properties of Corneal Epithelial Protein Extracts from *Pglyrp1*^{-/-} Mice

Bovine PGLYRP1 purified from granulocyte extracts has bacteriostatic and bactericidal properties.²¹ We sought to determine whether corneal epithelial PGLYRP1 has antibacterial properties. Corneal epithelial protein extracts from wild-type and *Pglyrp1* null mice were incubated with *E. coli* DH5α cultures in early log phase in 96-well plates. Bacterial colony-forming units were calculated at 60 and 120 minutes after inoculation (Fig. 4). The number of bacteria had almost doubled in the control wells not treated with epithelial protein extracts. In contrast, those treated with epithelial extracts from *Pglyrp1*^{+/+} mice showed no increase in the number of bacterial colony-forming units after 1 or 2 hours of incubation. Thus, the *Pglyrp1*^{+/+} corneal epithelial extract prevented bacterial growth. Moreover, this activity was lost on pretreatment of the extract with an anti-PGLYRP1 antibody. The *Pglyrp1*^{-/-} epithelial extract, on the other hand, showed little to no bacteriostatic activity. The results indicate that PGLYRP1 in the corneal epithelium has bacterial growth-deterrent activity.

Poor Healing of *Pseudomonas aeruginosa* Corneal Infection in *Pglyrp1*^{-/-} Mice

Given that *P. aeruginosa* is a common cause of bacterial infections of the cornea, we sought to determine whether *Pglyrp1* has a role in protecting the cornea against this bacterium. We used a mouse model of corneal scarification¹⁹ to compare *P. aeruginosa* infections of the cornea in *Pglyrp1*^{-/-} and *Pglyrp1*^{+/+} mice. Examination of the infected corneas showed considerable healing in the wild-type mice by 24 hours and completely healed corneas by 7 days (Fig. 5A). In contrast, the *Pglyrp1*^{-/-} mice began to show exacerbated signs of infection by 24 hours. To obtain a quantitative estimate of bacterial yield from infected corneas, wild-type and *Pglyrp1*^{-/-} mice (*n* = 6 per group) were challenged with *P. aeruginosa*. The mice were humanely killed after 5 days, and the corneas were homogenized to obtain total bacterial yield. Because the distributions of bacterial counts were highly skewed toward large values and control and knockout mice had substantially different scales, we used the nonparametric Wilcoxon rank sum test to test for a difference in means. The two-sided test was highly significant and gave a *P* value of 0.00866. Alternatively, a two-sided, two-sample *t*-test of the log-transformed data (in this case, one control sample with no bacterial counts was taken as 1 cfu) gave a *P* value of 0.006556 (Fig. 5B). Thus, there was a significantly higher yield of bacteria from corneal infections of *Pglyrp1*^{-/-} mice, indicating a compromise in their ability to fight *P. aeruginosa* corneal infections.

Delayed Healing of Corneal Wounds in *Pglyrp1*-Deficient Mice

The *Pglyrp* gene family is known to regulate host innate immune response and tissue homeostasis in insects.²² Little is known about whether mammalian *Pglyrp1* has a similar role in host innate immune response and cell survival signals. To address this question, we investigated the healing of noninfectious corneal epithelial wounds in *Pglyrp1*^{-/-} and

Pglyrp1^{+/+} mice. Circular corneal wounds were created using an Alger brush, as described,²⁰ and *S. aureus* peptidoglycan was added to the wounds. Observed under a fluorescence dissecting microscope, the *Pglyrp1*^{+/+} showed almost complete healing while remaining wounds were visible in the *Pglyrp1*^{-/-} mice (Fig. 6A). In the *Pglyrp1*^{-/-} mice, 17% to 29% of the original wound areas remained after 24 hours, as opposed to only 0% to 12% in the wild types (Fig. 6B).

Discussion

Our earlier genomewide gene expression study identified high transcript levels of a novel antimicrobial protein, *Pglyrp1*, in the mouse cornea.¹ As a follow-up to this initial observation, here we investigated its expression and role in the mouse cornea using several different approaches. First immunoblotting detected PGLYRP1 in total protein extracts from the mouse cornea. Further immunoblotting of corneal epithelial protein extracts showed the presence of this protein in the epithelium. By immunohistochemistry, we detected PGLYRP1 in the epithelial wing and superficial cells, with no detectable immunostaining of the basal undifferentiated epithelial layer. Immunostaining of sections of the human cornea also indicated its presence in the superficial epithelial layers.

To our knowledge, this is the first report of the PGLYRP1 antimicrobial protein at the ocular surface. There is only one other report of another member of this family, PGLYRP2 at the ocular surface. That study reported low levels of PGLYRP2 transcript in the cornea, and the *Pglyrp2* gene was inducible to low levels in a human corneal epithelial cell line in the presence of bacteria.¹⁶ Microbe-host interactions are beginning to be recognized as playing a directive role in maintaining an optimally active innate immune response and epithelial homeostasis in the host.²³ Similar microbial influence underpinning corneal epithelial homeostasis and regulation of *Pglyrp1* expression in the cornea by microbe-host interactions are unknown. Our results show constitutive high expression of PGLYRP1 in the mouse cornea, both at the mRNA and at the protein level. However, the present study and all previous reports on *Pglyrp1* have used mice raised in specific pathogen-free (SPF) facilities. The mucosal surfaces of SPF mice are colonized with commensal and some pathogenic bacteria. It is not known whether *Pglyrp1* is expressed equally well in mice, raised in germ-free facilities, free of all aerobic and anaerobic microbes.

Another point of speculation is how PGLYRP1 is stored or secreted by the epithelium. Epithelial secretion of antimicrobial proteins/peptides has been studied extensively in the gut. For example, intestinal Paneth cells make, and store large amounts of α defensins, which are released in the crypt in response to specific signals.^{24,25} PGLYRP3 and PGLYRP4 are expressed in the intestinal columnar absorptive epithelial cells, not Paneth cells, and increase in response to bacterial exposure.⁶ In the stratified corneal epithelium, the release of antimicrobial proteins/peptides may be different. PGLYRP1 may simply be released during natural sloughing off of the surface epithelial cells and their breakdown. On the other hand, PGLYRP1 has a signal peptide and may be secreted by surface epithelial cells in a regulated manner. At least in neutrophils it is known to be stored in tertiary granules. In activated neutrophils, it colocalizes with lysozyme in neutrophil extracellular traps and has a synergistic antibacterial effect with lysozyme.²⁶

To date, mammalian *Pglyrp1* has been associated with antibacterial functions only. In contrast, the insect *Pglyrp1* ortholog is known to be antibacterial and to cross-talk with *Toll* and other innate immune and survival signal-regulating genes. We found a small, but significant, delay in the healing of noninfectious corneal epithelial wounds in *Pglyrp1*^{-/-} mice. The epithelium initiates rapid healing of corneal wounds by downregulating apoptosis and promoting cell proliferation, growth, and migration.²⁷ Delayed healing of epithelial

wounds in *Pglyrp1*^{-/-} mice suggests a role for PGLYRP1 in modulating these epithelial cell survival and homeostatic signals. In addition, our study shows that, like the neutrophil-PGLYRP1, the corneal protein is also antibacterial. The wild type, but not the *Pglyrp1*^{-/-}, corneal epithelial protein extract prevented bacterial growth in culture. Bacteriostatic activity was lost on incubation of the *Pglyrp1*^{+/+} corneal extract with anti-PGLYRP1 antibody; confirming the bacteriostatic activity to be specifically caused by PGLYRP1. *P. aeruginosa* is a common cause of cornea infections in humans.²⁸ In our study, *Pglyrp1*^{-/-} mice showed exacerbated *P. aeruginosa* corneal infections and a failure to clear the bacteria, indicating a role for PGLYRP1 in antibacterial defense against this bacterium. PGLYRP1 may also have a role in epithelial wound healing signals, and this could secondarily affect healing of infectious wounds. An earlier study reported reduced survival of *Pglyrp1*^{-/-} mice challenged systemically with the Gram-positive bacterium *Bacillus subtilis*.⁴ Recombinant mammalian PGLYRP1 binds ly-sine-type Gram-positive and meso-diaminopimelic acid type Gram-negative bacterial peptidoglycans.²⁶ It can also recognize bacterial lipopolysaccharides and possibly pathogen-associated molecular patterns of fungal origin, suggesting a broad antimicrobial property.^{21,29}

Additional studies of PGLYRP1 are needed to elucidate its role in the cornea and to identify pathogen targets. Indeed, constitutively high levels of PGLYRP1 in the corneal epithelium may mean that this antimicrobial protein is a broad-spectrum resident pathogen deterrent at the ocular surface.

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Figure 1.

Immunoblot showing PGLYRP1 in mouse corneal protein extracts. **(A)** A single PGLYRP1 band is evident in total corneal extracts from *Pglyrp1*^{+/+} mice (lane 1) and bone marrow (lane 2) extracts. No PGLYRP1 staining was evident in the *Pglyrp1*^{-/-} mouse cornea (lane 3) or bone marrow extracts (lane 4). Actin immunoblotting shows equivalent loading of lanes. **(B)** Immunoblotting of total protein extracted from the corneal epithelium stripped away from the stroma and endothelium. Immunostaining of PGLYRP1 is evident in the *Pglyrp1*^{+/+} (+/+) and not *Pglyrp1*^{-/-} (-/-) corneal epithelial and bone marrow (BM) extracts.

Figure 2.

Immunohistology showing PGLYRP1 (*red*) expression in the mouse corneal epithelium by immunofluorescence confocal microscopy with an anti-PGLYRP1 antibody. The epithelium (Ep), stroma (S), and endothelium (En) are marked as shown. Cell nuclei were stained with DAPI (*blue*). Original magnifications: (A–H) 20 $\times$; (I–L) 100 $\times$. Scale bars: (A–H) 50 μ m; (I–L) 10 μ m.

Figure 3.

Immunohistology showing PGLYRP1 (*red*) expression in the human corneal epithelium. Immunofluorescence confocal microscopy with anti-PGLYRP1 antibody was performed to investigate the expression of PGLYRP1 in the human cornea. Cell nuclei were stained with DAPI (*blue*). Original magnifications: (A–H) 40×; (I–L) 100×. Scale bars: (A–H) 50 μ m; (I–L) 10 μ m.

Figure 4.

Corneal epithelial protein extract from *Pglyrp1*^{+/+} mice inhibit the growth of DH5α *E. coli* in 96-well plates. The data show the mean number of surviving bacteria cfu after 60 minutes and 120 minutes, in the presence and absence of 5 μg total corneal epithelial protein from *Pglyrp1*^{+/+} and *Pglyrp1*^{-/-} mice. Results shown are mean ± SEM of three wells. *Horizontal dotted line*: starting cfu at each time 0. The experiment shown is one of two similar experiments.

Figure 5. Delayed clearing of *P. aeruginosa* corneal infection in *Pglyrp1*^{-/-} mice. **(A)** A representative photograph of *P. aeruginosa* scarification infection model in *Pglyrp1*^{+/+} and *Pglyrp1*^{-/-} mice (four animals per genotype). A fluorescein dye was added to visualize the scratch wounds and healing 0 hour, 6 hours, 24 hours, and 7 days after bacterial inoculation. Note that after 7 days the *Pglyrp1*^{-/-} showed diffuse fluorescein staining and cloudy patches (arrow) indicative of bacterial infections, whereas the *Pglyrp1*^{+/+} cornea appeared healthy. **(B)** Bacterial yield from homogenized corneas 5 days after scarification and bacterial inoculation. Data points are mean of three measurements from each animal ($n = 6$ per genotype). $P = 0.00866$ (Wilcoxon rank sum test) or $P = 0.006556$ (two-sided, two-sample t -test of the log-transformed data, where one wild-type mouse with 0 cfu was taken as 1 cfu).

Figure 6.

Delayed wound healing in *Pglyrp1^{-/-}* compared to *Pglyrp1^{+/+}* mouse cornea. **(A)** Circular corneal wounds were photographed immediately (0 hour) or 24 hours after wounding (24 hours). Fluorescein dye (*green*) was used to detect wound healing using a fluorescein/FITC filter on a dissecting microscope, and the wound areas were measured. *Arrow*: unhealed wound left in the *Pglyrp1^{-/-}* mouse cornea. Corneas were also checked by bright-field microscopy with an external light source. The unwounded contralateral eye served as a control (not shown). **(B)** Graphical representation of healing shown as remaining wound area mean $\pm$ SEM ($n = 3$).

Table 1

Real-Time PCR Measurements of Relative Expression of *Pglyrp1* Compared with *Gapdh* mRNA in BALB/c Mouse Corneal Epithelial Cells

	<i>Pglyrp1</i>	<i>Gapdh</i>	Relative Expression ($2^{-\Delta CT}$)
C _T values	23.61	24.79	2.26
	23.58	24.74	2.23
	23.60	24.53	1.91
Average $\pm$ SD	23.59 $\pm$ 0.02	24.69 $\pm$ 0.14	2.13 $\pm$ 0.19