Histidine-rich glycoprotein exerts antibacterial activities

Victoria Rydengård [1], Anna-Karin Olsson [2], Matthias Mörgelin [3] and Artur Schmidtchen [1]

[1]Section of Dermatology and Venereology and [3] Section of Clinical and Experimental Infectious Medicine, Department of Clinical Sciences, Lund University, Biomedical Center, Tornavägen 10, SE-221 84 Lund, Sweden

[2]Department of Genetics and Pathology, Uppsala University, Rudbeck Laboratory, Dag Hammarskjölds väg 20, SE-751 85 Uppsala, Sweden

Running title: Antibacterial histidine-rich glycoprotein

Correspondence

Victoria Rydengård, Department of Clinical Sciences, Lund University, Biomedical Center B14, Tornavägen 10, S-221 84 Lund, Sweden, Tel: +46-46-2223315, Fax: +46-46-157756.

E-mail address: victoria.rydengard@med.lu.se

Abbreviations: AMP, antimicrobial peptide; HRGP, histidine-rich glycoprotein; HRR, histidine-rich region; Ni-NTA, nickel-nitrilotriacetic acid; HBP, heparin-binding protein; MES, 2-Morpholinoethanesulfonic acid; cfu, colony forming units

Keywords: Histidine-rich glycoprotein, antibacterial, pH, zinc, heparin

Histidine-rich glycoprotein (HRGP), an abundant heparin-binding protein found in plasma and thrombocytes, exerts antibacterial effects against Gram-positive bacteria (*Enterococcus faecalis* and *Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). Fluorescence studies and electron microscopy for assessement of membrane permeation demonstrated that HRGP induces lysis of *Enterococcus faecalis* bacteria in the presence of Zn^{2+} or low pH. Heparin blocked binding of the protein to *Enterococcus faecalis*, and abolished antibacterial activity. Furthermore, truncated HRGP, devoid of the heparin-binding and histidine-rich domain, was not antibacterial. It has previously been shown that peptides containing consensus heparin-binding sequences (Cardin and Weintraub motifs) are antibacterial. Thus, the peptide $(GHHPH)_4$, derived from the histidine-rich region of HRGP and containing such a heparin-binding motif, was antibacterial for *E. faecalis* in the presence of Zn^{2+} or low pH. The results demonstrate a previously undisclosed antibacterial activity of HRGP and suggest that the histidine-rich, and heparin-binding domain of HRGP mediates the antibacterial activity of the protein.

Introduction

Histidine-rich glycoprotein (HRGP) is an abundant 67 kDa plasma glycoprotein identified in many vertebrates and also in invertebrates, such as the blue mussel, *Mytilus edulis* [1, 2]. The protein is a member of the cystatin super family, along with kininogen, α-2-HS-glycoprotein and cystatin. HRGP is synthesized in the liver, found in plasma and is stored in the α-granules of thrombocytes, from which it is secreted upon thrombin activation [3]. The concentration in human plasma is ~ 2 μM but the local concentration, close to thrombocytes activated during

coagulation, is likely to be higher, reaching levels of 10-50 μM [4]. HRGP contains two cystatin-like domains, a central histidine-rich region (HRR) containing multiple GHHPH tandem repeats flanked by proline-rich regions, and a C-terminal region [4]. HRGP interacts, either via the HRR or other domains, with multiple ligands, such as heparan sulphate, heme, fibrinogen, thrombospondin, plasminogen, IgG, FcγR and C1q. Notably, the HRR of HRGP binds to heparan sulphate as well as heparin and this interaction is strongly enhanced in the presence of Zn^{2+} and at acidic conditions [4, 5].

Antimicrobial peptides (AMP) are important effector molecules of innate immunity [6]. AMPs interact with bacterial membranes, leading to membrane destabilization, intracellular changes and ultimately, bacterial killing [7-9]. Besides their antibacterial effects, additional biological effects exerted by AMPs include stimulation of growth and angiogenesis (angiogenins, LL-37), protease inhibition (SLPI), anti-angiogenesis (PR-39), and chemotaxis (chemokines, LL-37, defensins) [10, 11]. As recently shown by our group, AMPs and heparin-binding peptides **share many structural and functional features**. This applies to classical AMPs, such as LL-37 and defensin, as well as the anaphylatoxin C3a, domain 5 of high molecular weight kininogen, and also several other heparin-binding peptides derived from protein C inhibitor and other plasma and matrix proteins [12-14]. In conjunction with these findings, consensus heparin-binding peptide sequences (Cardin and Weintraub motifs) XBBBXXBX or XBBXBX (where X represents hydrophobic or uncharged amino acids, and B represents basic amino acids), represented by multiples of the motifs ARKKAAKA or AKKARA [15], were shown to be antibacterial [16] and specifically interact with membranes [17]. Furthermore, recent studies have shown that Cardin motif peptides having R and K replaced by H, thus yielding peptides containing the sequences AHHAHA and AHHHAAHA, are antibacterial in the presence of Zn^{2+} or in low pH [18].

The observation that the HRR of HRGP shares many functional (heparin binding and multifunctionality) and structural features (multiple GHHPH motifs resembling the heparin binding Cardin peptides), lead us to hypothesize that HRGP, or domains thereof, might possess antibacterial activities. Here we demonstrate that HRGP, as well as an HRR-derived peptide containing the GHHPH motif, exert pH and Zn^{2+} dependent antibacterial activities.

Results

Human serum HRGP was purified using Ni-NTA agarose and the molecular weight and purity of the protein was confirmed by SDS-PAGE and western blot (Fig. 1A). Aminoterminal sequencing of the ~70 kDa protein yielded the amino acids VSPTD, thus confirming the identity of the protein. Considering that the various activities of HRGP strongly depend on Zn^{2+} and pH, we wanted to investigate the influence of these factors on HRGP in relevant antibacterial assays. Initial experiments using viable count assays showed that various Gram-positive (*E. faecalis*, *S. aureus*) as well as Gram-negative (*P. aeruginosa*, *E. coli*) bacterial isolates were largely unaffected by low pH in the absence of HRGP. Only *E. faecalis* survived in the presence of 10-50 μM Zn^{2+} (data not shown), which was compatible with findings demonstrating that Zn^{2+} may exert antibacterial activity *per se*, especially against Gram-negative bacteria [19]. Thus, in order to evaluate both Zn^{2+} and pH effects on HRGP activity, we used *E. faecalis* as a test bacterium in the initial experiments. As demonstrated (Fig. 1B), HRGP was not antibacterial in 10 mM Tris buffer at pH 7.4. The protein exerted however, antibacterial effects in the same buffer supplemented with 50 μM

Zn^{2+} or in MES-buffer at pH 5.5. At a HRGP concentration of 0.6 μM and using various Zn^{2+} concentrations, it was demonstrated that 5 μM Zn^{2+} was required for antibacterial activity, and 50 μM Zn^{2+} was required for efficient bacterial killing (Fig. 1D). Having shown a prerequisite for Zn^{2+} for bacterial killing, we analysed the influence of Mg^{2+} and Ca^{2+} on the antibacterial activities of HRGP. As demonstrated in Fig. 1E, only the addition of Zn^{2+} significantly increased bacterial killing. **We also investigated the time dependence of bacterial killing. As demonstrated in Fig. 1C, approximately 50% of the *E. faecalis* bacteria were killed within 15 minutes in the presence of 3 μM HRGP. Complete killing was seen after 120 minutes in both the Zn^{2+} containing buffer and at pH 5.5.**

Next, the salt-dependence of the bacterial killing was investigated. As presented in Table 1, HRGP-mediated bacterial killing was partly inhibited at 50 mM NaCl, and 150 mM NaCl completely abrogated the antibacterial effects. We also investigated the effect of plasma proteins on the antibacterial action of HRGP. As demonstrated (Fig. 1F), HRGP (at 4 μM) retained its antibacterial activity in presence of 20% citrate plasma or serum, however, EDTA plasma appeared to inhibit the antibacterial effects. This is possibly due to the chelating effects of EDTA on the Zn^{2+} present in the plasma. It should be pointed out that the plasma and serum fractions were diluted in a low salt buffer (10 mM Tris, pH 7.4). Furthermore, as the concentration of HRGP in blood is ~2-3 μM, the final HRGP concentration in the experiments with serum or plasma was ~4.5 μM (4 μM purified HRGP combined with ~0.5 μM from serum and plasma). To exclude the possibility that contaminants in the HRGP preparation were responsible for the antibacterial activity of this molecule, recombinant human HRGP (rHRGP) was produced in human embryonic kidney cells and tested for antibacterial effects. As is the case for the purified HRGP, rHRGP was antibacterial against *E. faecalis,* and the activity was dependent on Zn^{2+} or low pH (Fig. 1G).

Next, we investigated the Zn^{2+}-dependent antibacterial effects of HRGP (at 3 and 30 μM) on different strains of *E. faecalis* (Fig. 2A). Two *E. faecalis* isolates (2374 and BD33/03) were effectively killed by 3 μM HRGP in Tris-buffer supplemented with 50 μM Zn^{2}, whereas at 30 μM, HRGP killed these two isolates irrespective of Zn^{2+}. 30 μM HRGP was required to kill *E. faecalis* ATCC 29212 and the effect was enhanced by Zn^{2+}. At pH 5.5, 3 μM of HRGP yielded >90% reduction of bacterial counts of *E. faecalis,* as well as *E. coli* and *P. aeruginosa* strains, whereas *S. aureus* was reduced by 50-70% (Fig. 2B). Finally, we compared the activity of HRGP with two other antimicrobial proteins/peptides; heparin binding protein (HBP) and histatin 5. *E. faecalis* was incubated with 6 μM HBP, histatin 5, or HRGP in 10 mM Tris buffer (with or without addition of 50 μM Zn^{2+}) or in 10 mM MES, pH 5.5. The three molecules exerted similar antibacterial effects in the presence of Zn^{2+} or at pH 5.5. Only HBP was antibacterial at pH 7.4 (Fig. 2C). The antibacterial activity of histatin 5 and HRGP was lost in 0.15 M NaCl (at pH 5.5), and no significant difference in activity was found between the two molecules. HBP retained ~50% of its antibacterial activity in 0.15 M NaCl (Fig. 2D).

Many AMPs kill bacteria by membrane lysis, others may translocate through membranes and subsequently interact with intracellular targets, such as DNA and mitochondria, resulting in bacterial killing [20, 21]. Electron microscopy analysis after negative staining of whole bacteria demonstrated that HRGP triggered membrane destabilization and release of cytoplasmic components (Fig. 3). This effect was only observed in 10 mM Tris, pH 7.4 in the presence of 50 μM Zn^{2+} (Fig. 3E) or in 10 mM MES, pH 5.5 (Fig. 3F), but not in these respective buffers without HRGP (Fig. 3A-C) or without Zn^{2+} (Fig. 3D). **These results were further substantiated using a LIVE/DEAD *Bac*Light bacterial viability kit to provide an**

indication of the fraction of live cells. As demonstrated in Fig. 4A, HRGP (1 μM) treated cells contained a significantly higher proportion of bacteria with permeabilized membranes, as compared to the controls. Intact membranes are impermeable to propidium iodide, thus influx of this dye is an indication of membrane permeation (Fig 4A). Fig. 4B (right panel) illustrates the increase in permeation obtained with 1 μM HRGP in Tris-buffer in presence of 50 μM Zn^{2+}. Taken together, these data strongly suggest that HRGP acts on bacterial membranes. The data does not however, demonstrate the exact mechanistic events mediated by HRGP, since secondary metabolic effects on bacteria may trigger bacterial death and membrane destabilization.

Irrespective of the exact and final mode of action, membrane binding is a prerequisite for the antibacterial action of a given antimicrobial peptide or protein. To investigate the binding of HRGP to bacteria, the protein was incubated with *E. faecalis* in presence of Zn^{2+}. The bacteria were pelleted by centrifugation and the binding of HRGP to bacterial cells was assessed by detection of HRGP in the bacterial pellet and supernatant. As demonstrated by western blot analysis, a significant fraction of added HRGP bound to *E. faecalis* (~50%, Fig. 5A). Furthermore, addition of heparin completely abolished the binding of HRGP to the bacteria in the pellet, indicating that the Zn^{2+} and heparin-binding HRR of HRGP mediates the interaction with *E. faecalis*. **In order to delineate the antimicrobial domains further, we analysed truncated HRGP (rHRGP1-240) in antibacterial assays. This form contains the two cystatin-like regions of HRGP, but is devoid of the HRR and C-terminal domains. The results showed (Fig. 5B) that had no antibacterial activity (at 0.6 μM) in the presence of Zn^{2+} or at pH 5.5. This is in contrast to the full-length recombinant HRGP (Fig. 5B). 6 μM rHRGP1-240 was not antibacterial (not shown). In this context, it should be mentioned that both rHRGP forms contain 6 aminoterminal histidine residues.**

Although this modification could be the reason for the slightly increased antibacterial activity of full-length HRGP (compare Fig. 1B with 1G), it did not apparently impose an antibacterial effect on the rHRGP1-240 form. Considering the well-known heparin binding capacity of HRR, its Zn^{2+} and pH dependence, as well as the absence of antibacterial activity for rHRGP1-240, it was plausible to focus on the HRR of HRGP. The HRR contains 12 tandem repeats of five consensus sequences of amino acids, GHHPH [4]. Notably, this motif is highly conserved among various vertebrate species (Fig. 5C). In order to determine whether this sequence motif binds to heparin, a 20-mer peptide $(GHHPH)_4$ (Fig. 5C) was synthesized and tested for heparin-binding (Fig. 4D) using an established slot-blot screening assay [12]. The results showed that the interaction between the GHH20 peptide and heparin was potentiated in the presence of Zn^{2+} and also at pH 5.5. Analogous results were obtained with HRGP (Fig. 4D). As demonstrated by fluorescence microscopy analysis, the GHH20 peptide **showed enhanced binding** to the bacterial cells in presence of Zn^{2+} (Fig. 4E), and the binding was completely blocked by heparin. In antibacterial assays the GHH20 peptide exerted antibacterial activities against *E. faecalis* 2374 in the presence of Zn^{2+} or at pH 5.5**, albeit at higher concentrations than those required for HRGP-mediated killing. The *E. coli* isolate was highy sensitive to GHH20 at pH 5.5 (Fig. 4F).**

Discussion

The main and novel finding in this report is that HRGP, an abundant plasma protein, exerts an antibacterial activity that is facilitated by low pH as well as the cation Zn^{2+}. In view of our results, it is reasonable to believe that this property of HRGP is a logical consequence of the

unique characteristics of the HRR of HRGP. Although the structure of HRGP has not yet been determined, **molecular modelling studies suggest that the HRR of HRGP forms a polyproline (II) helical structure with numerous imidazole binding units (within histidine residues) that protrude outward from the structural unit, with pairs of imidazoles forming the basic Zn^{2+} binding units [4, 22]. At physiological pH, HRGP is likely to remain negatively charged (pI 6.45). Due to its high content of histidine residues (~13%), which are concentrated to the HRR, it can acquire a positive charge either by incorporation of Zn^{2+}, or by protonation of histidine residues under acidic conditions [4]. The ability to become positively charged facilitates interactions between the HRR domain of HRGP and bacteria.** These results were substantiated by the finding that an evolutionary conserved region of HRGP containing the motif sequence GHHPH, was antibacterial in the presence of Zn^{2+}or low pH. It is of note, that a similar dependence on Zn^{2+} or low pH has been observed for various histidine-containing AMPs. This includes heparin binding sequences containing multiples of the sequences AHHAHA or AHHHAAHA, histatin 5 and peptides derived from histidine containing regions of domain 5 of human high molecular weight kininogen [18] as well as antimicrobial histidine-rich peptides of tunicates (clavanins) [23] and the ergot fungus *Verticillium kibiense* [24]. Furthermore, poly-L-histidine, as well as the the 25-mer peptide $(GHHPH)_5$ of HRGP has been shown to bind and neutralise LPS [25]. Although the focus in that study was on endotoxin-neutralization, it is interesting to note that LPS is the major constituent of Gram-negative cell walls, and hence, the observation is compatible with our presented findings on the antibacterial activity of the GHH20 peptide. Clearly, several lines of evidence point to the HRR of HRGP as one effector of antimicrobial activity. However, the data does not rule out the presence of other antimicrobial regions in the protein, or that conformational changes mediated by HRR interactions, lead to the exposure of additional antimicrobial epitopes within the molecule.

It is well established that many AMPs are generated by proteolysis of larger, and non-antimicrobial holoproteins, and this illustrates a common theme in innate immunity. For example, the cathelicidin LL-37 is released from hCAP18, and other AMPs are proteolytically generated from complement factor C3 and high molecular weight kininogen [6, 13, 14]. Interestingly, the fact that HRGP efficiently kills bacteria, indicates that proteolysis of HRGP is not required for antibacterial activity. Notably, like HRGP, several antimicrobial proteins exert antimicrobial functions *per se*, and this includes bacterial permeability increasing protein, serprocodins such as proteinase 3, elastase and HBP (used herein for comparison), as well as lactoferrin [26].

Antibacterial proteins, such as BPI and lactoferrin, may give rise to peptides exerting antibacterial activities [27, 28]. Clearly, the possibility that HRGP may release antibacterial peptides needs to be addressed in future studies. The fact that the HRGP-derived peptide GHH20 was antibacterial, exemplifies that the holoprotein is not a prerequisite for antibacterial action. Interestingly, data are emerging that proteolysis of HRGP may generate bioactive fragments involved in antiangiogenesis [22, 29]. Analogously, recent data indicate that human plasmin, as well as human neutrophil enzymes such as elastase, efficiently degrade HRGP, yielding peptides containing the GHH20-epitope (Rydengård, in preparation). This represents a proof of the concept that endogenously produced peptides of HRGP may indeed function as AMPs.

Considering the influence of pH and Zn^{2+} on the antibacterial activity of HRGP, it is interesting to note that similar Zn^{2+} and pH dependence has been demonstrated for many ligands of HRGP, such as cell surface heparan sulphate and tropomyosin [30, 31]. Indeed, it

has been proposed that HRGP acts as a pH and Zn^{2+} sensor, providing a mechanism for the regulation of the various activities of HRGP, such as antiangiogenesis [5, 32]. Therefore, it is interesting to note that organs such as the skin have a low pH (pH~ 5), and that acidic conditions are likely to occur in other biological fluids following oxidative burst response of leukocytes [33]. Furthermore, it is of note that the total concentration of Zn^{2+} in plasma is 10-18 μM, but that thrombocytes can accumulate levels of Zn^{2+} 25 to 60-fold higher than those found in plasma [34]. Additionally, human skin has been reported to contain significant levels of Zn^{2+} (~0.5 mM) [35]. Although many properties have been ascribed to HRGP, few data are available on its possible *in vivo* role**. It was *not* within the scope of this work to prove a physiological role for HRGP in innate defence. Clearly, it remains to be investigated whether the herein disclosed novel antibacterial activity of HRGP also implicates a true antibacterial function for this protein, or fragments thereof, *in vivo*. The finding that physiological salt abrogated the antibacterial effect of HRGP clearly challenges the hypothesis that the protein may exert antibacterial functions *in vivo*. Nevertheless, it must be noted that many AMPs, which have potent bactericidal activities *in vitro*, are antagonized by physiological salt, or the presence of plasma or serum [36]. For example, the extensively studied histidine-rich AMP histatin 5, showed a similar loss of activity in the presence of 0.15 M NaCl. Other groups have also shown that the cathelicidin LL-37 and also HBP are inhibited in the presence of serum or plasma [37, 38]. It may therefore be speculated that compartmentalization of AMPs, the presence of ionic microenvironments, and synergism between AMPs in specific environments, may facilitate a controlled antimicrobial action for a given antimicrobial factor *in vivo*. In this context, it is interesting to note that HRGP binds avidly to fibrin clots [39].** These physiologically important "barriers", formed during hemostasis and infection, constitute a unique milieu with high levels of surface-immobilized HRGP. Current investigations aim to

explore the possible antimicrobial functions of HRGP in clots *in vivo*. Of relevance to this is the finding that clots generated from normal human plasma exert significantly stronger antimicrobial effects than those observed for HRGP-depleted fibrin clots (Rydengård, in preparation). Recent data indicate that mice lacking HRGP show enhanced blood coagulation and fibrinolysis [40]. It remains to be investigated whether these animals also have a compromised innate immune response.

Experimental procedures

Materials

The peptide GHH20 (GHHPHGHHPHGHHPHGHHPH) was synthesized by Innovagen AB (Lund, Sweden). The purity and molecular weight was confirmed by MALDI-TOF MS analysis (Voyager, Applied Biosystems**).** Histatin 5 (DSHAKRHHGYKRKFHEKHHSHRGPY) was a generous gift from Dr. Martin Malmsten, Uppsala University. Heparin binding protein (HBP) was kindly provided by Dr. Heiko Herwald, Lund University. Polyclonal rabbit antibodies against GHH20 and TAMRA labelled GHH20 peptide were purchased from Innovagen AB (Lund, Sweden). The Gram-positive *E. faecalis* 2374, *E. faecalis* BD 33/03, *S. aureus* 80, *S. aureus* BD 312 and Gram-negative *E. coli* 37.4, *E. coli 47.1, P. aeruginosa* 27.1 and *P. aeruginosa* 15159 were all clinical isolates. *E. faecalis* ATCC 29212, *S. aureus* ATCC 29213, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 isolates were from the American Type Culture Collection (ATCC, Rockville, MD). Human serum and plasma was collected from healthy volunteers. For preparation of

EDTA plasma, vacutainer tubes containing K3EDTA (2 mg/ml, BD Biosciences, San Jose, CA, USA) were used. Citrate plasma was prepared using vacutainer tubes containing 1:9 (v/v) 129 mM sodium citrate.

Purification of human HRGP

Serum HRGP was purified according to Mori *et al*, with minor changes [41]. Human blood was incubated at room temperature for 1 h, and then centrifuged at 210 x g for 15 minutes. Twenty ml serum was aspirated and gently shaken at 4ºC together with 2 ml nickel-nitrilotriacetic acid (Ni-NTA) agarose overnight, applied onto a column, and washed with 30 volumes of 10 mM phosphate-buffered saline (PBS, pH 7.4). Proteins bound nonspecifically to the column were eluted with 10 volumes of PBS, containing 80 mM imidazole. Finally, HRGP was eluted in PBS supplemented with 500 mM imidazole. The protein was dialyzed against 2 mM NH_4HCO_3, freeze-dried and then resuspended in distilled water. The concentration of the protein was determined using the Bradford method [42]. Protein sequence analysis was carried out at the Protein Analysis Center, Karolinska Institutet, Stockholm, Sweden. Edman degradation was performed after concentration and clean-up of the protein solution using a Prosorb sample preparation cartridge (Applied Biosystems, Foster City, CA) according to the manufacturers instruction but without addition of BioBrene Plus to the PVDF membrane before application to a Procise cLC or a Procise HT sequencer instrument (Applied Biosystems, Foster City, CA).

Production and purification of recombinant HRGP (rHRGP and rHRGP1-240)

Full length cDNA encoding human HRGP was cloned into the pCEP-Pu2 expression vector [43]. The truncated version of HRGP containing amino acids 1-240 (HRGP1-240, also previously referred to as His2 [29] was produced by PCR-amplification. A His-tag was

introduced at the N-terminal end of the HRGP coding region, to enable purification. The signal sequence derived from the HRGP gene was excluded and the sequence containing His-tagged HRGP was instead ligated in frame with the BM40 signal sequence in pCEP-Pu2. An enterokinase cleavage site was introduced between the His-tag and the HRGP coding region, but was never used due to enterokinase spuriously cleaving within the HRGP polypeptide chain. HEK 293-EBNA cells were used to produce recombinant HRGP. These cells are stably transfected with the EBNA-1 gene, which is also expressed by the pCEP-Pu2 vector, thereby preventing chromosomal integration of transfected plasmid DNA. This allows an overall high yield of recombinant protein. The HRGP expression vectors were transfected using Lipofectamine™ (Invitrogen) and selected with 2.5 μg/ml puromycin (Sigma). In order to avoid contamination with bovine HRGP, a defined serum-replacement medium, TCM™ (ICN Biomedicals) was used instead of FCS for collection of conditioned medium. His-tagged HRGP was purified from conditioned medium using Ni-NTA agarose as described above. It is important to note that both rHRGP forms contain 6 aminoterminal histidine residues not found in the endogenous protein.

Western blot

Purified HRGP (~70 ng) was run on 8% SDS-polyacrylamide gels (SDS-PAGE), and subsequently transferred to a nitrocellulose membrane (Hybond-C, GE Healthcare BioSciences, Little Chalfont, UK)[44]. The membrane was incubated with 3% skimmed milk in 10 mM Tris, 0.15 M NaCl, pH 7.4 for 1 hour at room temperature, followed by an incubation for 1 hour with rabbit polyclonal antibodies against GHH20 (diluted 1:1000 in the same buffer). The membrane was washed 3 times, and incubated for 1 hour with horseradish peroxidase-conjugated secondary swine anti rabbit antibodies diluted 1:1000 (Dako, Carpinteria, CA). The image was developed using the ECL system (Amersham Biosciences).

Initial studies using western blot analysis showed that the GHH-antibodies specifically recognized HRGP, since; (a) No immunoreactive signal was detected in HRGP-depleted plasma, (b) pre-immune serum did not recognize any HRGP, (c) the antibodies did not bind to the related domain 5 of high molecular weight kininogen.

Viable count assay

E. faecalis, *S. aureus*, *E. coli* and *P. aeruginosa* bacteria were grown to mid-logarithmic phase in Todd-Hewitt (TH) medium (Becton and Dickinson, Maryland, USA) and washed in 10 mM Tris, pH 7.4 or 10 mM 2-Morpholinoethanesulfonic acid (MES), pH 5.5. For dose-response experiments, purified serum HRGP (0.003-3 μM), rHRGP (0,03-1.2 μM) or GHH20 (0.03-60 μM) were incubated with 1 x 10^5 *E. faecalis* 2374 or *E. coli* 80 for 2 hours at 37^{o}C in 10 mM, Tris, pH 7.4 (with or without 50 μM Zn^{2+}), or in 10 mM MES-buffer, pH 5.5. The samples were then plated on TH agar and the number of colony forming units (cfu) was determined. In order to determine the antibacterial effect of 0.6 μM HRGP at various Zn^{2+}concentrations, bacteria were incubated in 10 mM Tris, pH 7.4, containing 0, 5, 10, 25 or 50 μM Zn^{2+}. In order to investigate the effects of diverse cations on the activity of HRGP, 0.6 μM HRGP was incubated with *E. faecalis* 2374 in 10 mM Tris, pH 7.4 supplemented with 50 μM Zn^{2+}, Mg^{2+} or Ca^{2+}, and the number of cfu was determined. In order to investigate the antibacterial activity of HRGP in the presence of plasma proteins, *E. faecalis* 2374 was incubated with HRGP (4 μM) in 10 mM Tris, pH 7.4, containing EDTA-plasma, citrate plasma or serum (all at 20%), and the number of cfu was determined. In order to determine the antibacterial effect of HRGP in the presence of NaCl, *E. faecalis* 2374 was incubated with 0.6 μM HRGP in 10 mM Tris, pH 7.4, 50 μM Zn^{2+}, or 10 mM MES, pH 5.5 with addition of 0, 25, 50, 100, or 150 mM NaCl. In order to assess possible strain variation for the antibacterial effects, antibacterial assays were performed using *E. faecalis* 2374, *E. faecalis*

BD 33/03 and *E. faecalis* ATCC 29212 in the presence of 3 and 30 μM HRGP for 2 h in 10 mM Tris pH 7.4 (with or without 50 μM Zn^{2+}). The antibacterial effect of HRGP against various Gram-positive and Gram-negative bacteria in low pH-buffer was determined (as indicated in Fig. 2B), by incubating 3 μM HRGP with bacteria in 10 mM Tris, pH 7.4 or 10 mM MES, pH 5.5, and plating on TH agar for cfu determination. The antibacterial effect of HRGP was compared with other known antimicrobial (poly)peptides, by incubating *E. faecalis* 2374 with 0.03-6 μM HBP, histatin 5 or HRGP in 10 mM Tris-buffer pH 7.4 (with or without 50 μM Zn^{2+}) or in 10 mM MES, pH 5.5, followed by plating on TH agar for cfu determination. The antibacterial effects of HBP, histatin 5 and HRGP were also investigated in physiological salt. *E. faecalis* 2374 was incubated with HBP, histatin 5 and HRGP (all at 6 μM) for two hours in 10 mM MES, pH 5.5 with addition of 0.15M NaCl, and the number of cfu was determined. In experiments with truncated recombinant HRGP (0.6 μM rHRGP or rHRGP1-240 was incubated with *E. faecalis* 2374 for 2 hours in 10 mM Tris, pH 7.4 containing 50 μM Zn^{2+} or 10 mM MES, pH 5.5. In all experiments, 100% survival was defined as total survival of bacteria in the same buffer and under the same conditions in the absence of peptide or protein. The p-value was determined using Kruskall-Wallis one-way ANOVA analysis.

Binding of histidine-rich glycoprotein to bacteria

E. faecalis (1 x 10^5 bacteria) was incubated with 0.6 μM HRGP in 50 μl 10 mM Tris, pH 7.4 containing 50 μM Zn^{2+}, in the presence or absence of heparin (50 μg/ml) for 2 hours at 37°C, centrifuged and the pellet was washed three times with 10 mM Tris, pH 7.4. The pellet and the supernatant were resuspended in SDS sample buffer, run on an 8% SDS-PAGE, and then transferred to a nitrocellulose membrane. Western blotting was performed as previously described.

Heparin-binding assay

The radioiodination of heparin (from porcine intestinal mucosa, Sigma) was performed according to previous protocols [45, 46]. Two and 5 μg of GHH20 or purified serum HRGP was applied to nitrocellulose membranes (Hybond-C) using a slot blot apparatus. The membranes were incubated with ^{125}I radiolabelled heparin (10 μg/ml) for 1 hour at room temperature in 10 mM Tris, pH 7.4 (with or without 50 μM Zn^{2+}), or in 10 mM MES, pH 5.5. The membranes were washed 3 x 10 minutes in the corresponding buffer. A Bas 2000 radio imaging system (Fuji) was used to visualize the radioactive signal.

Fluorescence microscopy

E. faecalis was grown in TH medium at 37ºC to the mid-logarithmic phase of growth. The bacteria were washed in 10 mM Tris, pH 7.4, and resuspended in the same buffer. *E. faecalis* (2 x 10^6/ ml) was incubated with 2 μg of TAMRA-labelled GHH20 (2 mg/ml) in 10 mM Tris, pH 7.4 or 10 mM Tris, pH 7.4 containing 50 μM Zn^{2+}, in the absence or presence of heparin (50 μg/ml). After 5 minutes on ice the samples were washed twice in 10 mM Tris, pH 7.4. Bacteria were fixed with 4% paraformaldehyde by incubation on ice for 15 minutes and at room temperature for 45 minutes. Glass cover-slips were coated with 50 μg Poly-L-lysine (70-150 kDa) diluted in distilled water. The bacteria were applied onto the coated cover slips and after 30 minutes the samples were mounted on slides using Dako mounting media (Dako, Carpinteria, CA). Samples were visualized using a Nikon Eclipse TE300 inverted fluorescence microscope equipped with a Hamamatsu C4742-95 cooled CCD camera, a Plan Apochromat 100X objective and a high N.A. oil condenser.

Negative staining and transmission electron microscopy

E. faecalis bacteria were grown in TH medium at 37ºC to the mid-logarithmic phase of growth. The bacteria were washed in 10 mM Tris, pH 7.4 (with or without 50 μM Zn^{2+}) or 10 mM MES, pH 5.5 and resuspended in the same buffer. HRGP or LL-37 (10 μM) was incubated with *E. faecalis* (**2 x 10^6 bacteria**) for two hours in a total volume of 10 μl in Tris buffer, pH 7.4 (with or without Zn^{2+}) or in MES buffer, pH 5.5. Samples of *E. faecalis* bacteria suspensions were adsorbed onto carbon-coated copper grids for 1 minute, washed briefly with water, and negatively stained with 0.75 % uranyl formate. The grids were rendered hydrophilic by glow discharge at low pressure in air. Specimens were examined in a Jeol JEM 1230 electron microscope operated at 60 kV accelerating voltage and images were recorded with a Gatan Multiscan 791 CCD camera.

Bacterial viability

E. faecalis 2374 (1 x 10^7 bacteria) was incubated with 1 μM HRGP in 10 mM Tris, pH containing 50 μM Zn^{2+} or 10 mM MES, pH 5.5 for two hours. After incubation the samples were analysed using a LIVE/DEAD Baclight Bacterial Viability Kit, according to the manufacturers instructions (Invitrogen, Carlsbad, CA). Briefly, this kit utilises two fluorescent nucleic acid stains: SYTO9 and propidium iodide. SYTO9 (excitation and emission maxima, 480 and 500 nm) penetrates both viable and nonviable bacteria, while propidium iodide (excitation and emission maxima, 490 and 635 nm) penetrates only those bacteria that have damaged plasma membranes and quenches the SYTO9 fluorescence. Bacterial cells with compromised membranes fluoresce red and those with intact membranes fluoresce green. After incubation, bacteria samples were examined using a Nikon Eclipse TE300 inverted fluorescence microscope equipped with a Hamamatsu C4742-95 cooled CCD camera, a Plan

Apochromat 100X objective and a high N.A. oil condenser. The results were expressed as the percentage of killed bacteria.

Acknowledgements

This research was supported by grants from from the Swedish Research Council (projects 13471), the Royal Physiographic Society in Lund, the Söderberg, Schyberg, Welander-Finsen, Crafoord, Österlund, Lundgrens, Lions and Kock Foundations, DermaGen AB, and The Swedish Government Funds for Clinical Research (ALF). We wish to thank Ms. Maria Baumgarten for expert technical assistance and Dr. Oonagh Shannon for language revision.

References

1. Nair, P. S. & Robinson, W. E. (1999) Purification and characterization of a histidine-rich glycoprotein that binds cadmium from the blood plasma of the bivalve Mytilus edulis, *Arch Biochem Biophys. 366*, 8-14.
2. Shigekiyo, T., Yoshida, H., Matsumoto, K., Azuma, H., Wakabayashi, S., Saito, S., Fujikawa, K. & Koide, T. (1998) HRG Tokushima: molecular and cellular characterization of histidine-rich glycoprotein (HRG) deficiency, *Blood. 91*, 128-33.
3. Leung, L. L., Harpel, P. C., Nachman, R. L. & Rabellino, E. M. (1983) Histidine-rich glycoprotein is present in human platelets and is released following thrombin stimulation, *Blood. 62*, 1016-21.
4. Jones, A. L., Hulett, M. D. & Parish, C. R. (2005) Histidine-rich glycoprotein: A novel adaptor protein in plasma that modulates the immune, vascular and coagulation systems, *Immunol Cell Biol. 83*, 106-18.
5. Vanwildemeersch, M., Olsson, A. K., Gottfridsson, E., Claesson-Welsh, L., Lindahl, U. & Spillmann, D. (2006) The anti-angiogenic His/Pro-rich fragment of histidine-rich glycoprotein binds to endothelial cell heparan sulfate in a Zn2+-dependent manner, *J Biol Chem*.
6. Zasloff, M. (2002) Antimicrobial peptides of multicellular organisms, *Nature. 415*, 389-95.
7. Schröder, J. M. & Harder, J. (1999) Human beta-defensin-2, *Int J Biochem Cell Biol. 31*, 645-51.

8. Boman, H. G. (2000) Innate immunity and the normal microflora, *Immunol Rev. 173*, 5-16.
9. Zasloff, M. (1992) Antibiotic peptides as mediators of innate immunity, *Curr Opin Immunol. 4*, 3-7.
10. Gallo, R. L., Murakami, M., Ohtake, T. & Zaiou, M. (2002) Biology and clinical relevance of naturally occurring antimicrobial peptides, *J Allergy Clin Immunol. 110*, 823-31.
11. Zanetti, M. (2004) Cathelicidins, multifunctional peptides of the innate immunity, *J Leukoc Biol. 75*, 39-48.
12. Andersson, E., Rydengård, V., Sonesson, A., Mörgelin, M., Björck, L. & Schmidtchen, A. (2004) Antimicrobial activities of heparin-binding peptides, *Eur J Biochem. 271*, 1219-26.
13. Nordahl, E. A., Rydengård, V., Nyberg, P., Nitsche, D. P., Mörgelin, M., Malmsten, M., Björck, L. & Schmidtchen, A. (2004) Activation of the complement system generates antibacterial peptides, *Proc Natl Acad Sci U S A. 101*, 16879-84.
14. Nordahl, E. A., Rydengård, V., Mörgelin, M. & Schmidtchen, A. (2005) Domain 5 of high molecular weight kininogen is antibacterial, *J Biol Chem. 280*, 34832-9.
15. Cardin, A. D. & Weintraub, H. J. (1989) Molecular modeling of protein-glycosaminoglycan interactions, *Arteriosclerosis. 9*, 21-32.
16. Andersson, E., Rydengard, V., Sonesson, A., Morgelin, M., Bjorck, L. & Schmidtchen, A. (2004) Antimicrobial activities of heparin-binding peptides, *Eur J Biochem. 271*, 1219-26.
17. Ringstad, L., Schmidtchen, A. & Malmsten, M. (2006) Effect of peptide length on the interaction between consensus peptides and DOPC/DOPA bilayers, *Langmuir. 22*, 5042-50.
18. Rydengård, V., Andersson Nordahl, E. & Schmidtchen, A. (2006) Zinc potentiates the antibacterial effects of histidine-rich peptides against Enterococcus faecalis, *Febs J. 273*, 2399-406.
19. Aarestrup, F. M. & Hasman, H. (2004) Susceptibility of different bacterial species isolated from food animals to copper sulphate, zinc chloride and antimicrobial substances used for disinfection, *Vet Microbiol. 100*, 83-9.
20. Boman, H. G., Agerberth, B. & Boman, A. (1993) Mechanisms of action on Escherichia coli of cecropin P1 and PR-39, two antibacterial peptides from pig intestine, *Infect Immun. 61*, 2978-84.
21. Den Hertog, A. L., Wong Fong Sang, H. W., Kraayenhof, R., Bolscher, J. G., Van't Hof, W., Veerman, E. C. & Nieuw Amerongen, A. V. (2004) Interactions of histatin 5 and histatin 5-derived peptides with liposome membranes: surface effects, translocation and permeabilization, *Biochem J. 379*, 665-72.
22. Borza, D. B., Tatum, F. M. & Morgan, W. T. (1996) Domain structure and conformation of histidine-proline-rich glycoprotein, *Biochemistry. 35*, 1925-34.
23. Lee, I. H., Cho, Y. & Lehrer, R. I. (1997) Effects of pH and salinity on the antimicrobial properties of clavanins, *Infect Immun. 65*, 2898-903.
24. Nishikawa, M. & Ogawa, K. (2004) Antimicrobial activity of a chelatable poly(arginyl-histidine) produced by the ergot fungus Verticillium kibiense, *Antimicrob Agents Chemother. 48*, 229-35.
25. Bosshart, H. & Heinzelmann, M. (2003) Endotoxin-neutralizing effects of histidine-rich peptides, *FEBS Lett. 553*, 135-40.
26. Levy, O. (2000) Antimicrobial proteins and peptides of blood: templates for novel antimicrobial agents, *Blood. 96*, 2664-72.
27. Elsbach, P. & Weiss, J. (1998) Role of the bactericidal/permeability-increasing protein in host defence, *Curr Opin Immunol. 10*, 45-9.
28. Gifford, J. L., Hunter, H. N. & Vogel, H. J. (2005) Lactoferricin: a lactoferrin-derived peptide with antimicrobial, antiviral, antitumor and immunological properties, *Cell Mol Life Sci. 62*, 2588-98.

29. Olsson, A. K., Larsson, H., Dixelius, J., Johansson, I., Lee, C., Oellig, C., Björk, I. & Claesson-Welsh, L. (2004) A fragment of histidine-rich glycoprotein is a potent inhibitor of tumor vascularization, *Cancer Res. 64*, 599-605.
30. Mori, S., Shinohata, R., Renbutsu, M., Takahashi, H. K., Fang, Y. I., Yamaoka, K., Okamoto, M., Yamamoto, I. & Nishibori, M. (2003) Histidine-rich glycoprotein plus zinc reverses growth inhibition of vascular smooth muscle cells by heparin, *Cell Tissue Res. 312*, 353-9.
31. Guan, X., Juarez, J. C., Qi, X., Shipulina, N. V., Shaw, D. E., Morgan, W. T., McCrae, K. R., Mazar, A. P. & Donate, F. (2004) Histidine-Proline Rich Glycoprotein (HPRG) binds and transduces anti-angiogenic signals through cell surface tropomyosin on endothelial cells, *Thromb Haemost. 92*, 403-12.
32. Borza, D. B. & Morgan, W. T. (1998) Histidine-proline-rich glycoprotein as a plasma pH sensor. Modulation of its interaction with glycosaminoglycans by ph and metals, *J Biol Chem. 273*, 5493-9.
33. Wright, J., Schwartz, J. H., Olson, R., Kosowsky, J. M. & Tauber, A. I. (1986) Proton secretion by the sodium/hydrogen ion antiporter in the human neutrophil, *J Clin Invest. 77*, 782-8.
34. Gorodetsky, R., Mou, X., Blankenfeld, A. & Marx, G. (1993) Platelet multielemental composition, lability, and subcellular localization, *Am J Hematol. 42*, 278-83.
35. Michaelsson, G., Ljunghall, K. & Danielson, B. G. (1980) Zinc in epidermis and dermis in healthy subjects, *Acta Derm Venereol. 60*, 295-9.
36. Brown, K. L. & Hancock, R. E. (2006) Cationic host defense (antimicrobial) peptides, *Curr Opin Immunol. 18*, 24-30.
37. Gabay, J. E., Scott, R. W., Campanelli, D., Griffith, J., Wilde, C., Marra, M. N., Seeger, M. & Nathan, C. F. (1989) Antibiotic proteins of human polymorphonuclear leukocytes, *Proc Natl Acad Sci U S A. 86*, 5610-4.
38. Wang, Y., Agerberth, B., Lothgren, A., Almstedt, A. & Johansson, J. (1998) Apolipoprotein A-I binds and inhibits the human antibacterial/cytotoxic peptide LL-37, *J Biol Chem. 273*, 33115-8.
39. Leung, L. L. (1986) Interaction of histidine-rich glycoprotein with fibrinogen and fibrin, *J Clin Invest. 77*, 1305-11.
40. Tsuchida-Straeten, N., Ensslen, S., Schafer, C., Woltje, M., Denecke, B., Moser, M., Graber, S., Wakabayashi, S., Koide, T. & Jahnen-Dechent, W. (2005) Enhanced blood coagulation and fibrinolysis in mice lacking histidine-rich glycoprotein (HRG), *J Thromb Haemost. 3*, 865-72.
41. Mori, S., Takahashi, H. K., Yamaoka, K., Okamoto, M. & Nishibori, M. (2003) High affinity binding of serum histidine-rich glycoprotein to nickel-nitrilotriacetic acid: the application to microquantification, *Life Sci. 73*, 93-102.
42. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal Biochem. 72*, 248-54.
43. Vernersson, M., Ledin, A., Johansson, J. & Hellman, L. (2002) Generation of therapeutic antibody responses against IgE through vaccination, *Faseb J. 16*, 875-7.
44. Laemmli, U. K., Beguin, F. & Gujer-Kellenberger, G. (1970) A factor preventing the major head protein of bacteriophage T4 from random aggregation, *J Mol Biol. 47*, 69-85.
45. Cheng, F., Yoshida, K., Heinegard, D. & Fransson, L. A. (1992) A new method for sequence analysis of glycosaminoglycans from heavily substituted proteoglycans reveals non-random positioning of 4- and 6-O-sulphated N-acetylgalactosamine in aggrecan-derived chondroitin sulphate, *Glycobiology. 2*, 553-61.

46. Schmidtchen, A., Frick, I-M, and Björck, L. (2001) Dermatan sulfate is released by proteinases of common pathogenic bacteria and inactivates antibacterial alpha-defensin, *Molecular Microbiology*. *39*, 708-713.

Table 1. Effects of salt on HRGP activity**.** *E. faecalis* bacteria were incubated with 3 μM HRGP in 10 mM Tris, pH 7.4 containing 50 μM Zn^{2+} or in 10 mM MES, pH 5.5 in presence of the indicated concentrations of NaCl. Results are expressed as % survival (mean values are indicated, n=2).

Buffer	NaCl (mM)				
	0	25	50	100	150
10 mM Tris, pH 7.4 with 50μM Zn^{2+}	0	4	64	66	100
10 mM MES, pH 5.5	0	59	65	100	100

Figure legends

Fig. 1. Purification and antibacterial effects of histidine-rich glycoprotein**.**

(A) Left panel; SDS-PAGE analysis of material obtained from the purification steps on Nickel-agarose. 1; human serum, 2; material from first PBS washing step, 3; material eluted with 80 mM imidazole in PBS, 4; protein eluted with 0.5 M imidazole in PBS. Right panel; Detection of HRGP by western blotting. Material eluted from the Nickel-agarose column by 0.5 M imidazole was run on SDS-PAGE and transferred to a nitrocellulose membrane. Western blot was performed using polyclonal antibodies directed against the GHH20 peptide of HRGP. (B) Antibacterial effect of human serum HRGP. Purified human HRGP at concentrations ranging from 0.003 to 3 μM was incubated with 1 x 10^5 *E. faecalis* 2374 bacteria for 2 hours in 10 mM Tris, pH 7.4 (●), 10 mM Tris, pH 7.4, 50 μM Zn^{2+} (○) or 10 mM MES, pH 5.5 (▼), plated and the number of cfu determined. A representative experiment (of three) is shown. (C) Antibacterial activity of HRGP at various Zn^{2+} concentrations. Viable

count analysis was performed using *E. faecalis* 2374 bacteria incubated with 0.6 μM HRGP in 10 mM Tris, pH 7.4 at the indicated Zn^{2+} concentrations. After incubation the samples were plated and the number of cfu was determined (n=6). (D) Viable count analysis of HRGP in the presence of different divalent ions. 0.6 μM HRGP was incubated with 1 x 10^5 *E. faecalis* bacteria in 10 mM Tris, pH 7.4 with addition of 50 μM Zn^{2+}, Mg^{2+}, or Ca^{2+}. Identical buffers without HRGP were used as control (labelled with C). Significance was determined using Kruskall-Wallis one-way ANOVA analysis (*** $p<0.001$, n=6). (E) Killing kinetics. *E. faecalis* 2374 was incubated with HRGP (3 μM) in 10 mM Tris, pH 7.4 containing 50 μM Zn^{2+} (black bars) or 10 mM MES, pH 5.5 (grey bars) for 15, 30, 60 or 120 minutes. After incubation the samples were plated and the number of cfu was determined (n=6). (F) Antibacterial effects of HRGP in presence of human plasma proteins. *E. faecalis* was incubated with HRGP (at 4 μM) in 10 mM Tris, pH 7.4 containing 20% of the indicated plasma fractions and number of cfu was determined. Identical buffers without HRGP were used as control (labelled with C). Significance was determined using Kruskall-Wallis one-way ANOVA analysis (*** $p<0.001$, n=6). (G) Antibacterial effect of recombinant HRGP (rHRGP). In viable count assays, rHRGP at concentrations ranging from 0.03 to 1.2 μM was incubated with *E. faecalis* in 10 mM Tris, pH 7.4 (●), 10 mM Tris, 50 μM Zn^{2+}, pH 7.4 (○) or 10 mM MES, pH 5.5 (▼) and the number of cfu was determined. A representative experiment (of three) is shown. When indicated, error bars represent SD.

Fig. 2. Antibacterial activity of HRGP against different strains of Gram-positive and Gram-negative bacteria and comparison with HBP and histatin 5. (A) Antibacterial activity of HRGP against *E. faecalis* 2374, BD 33/03 or ATCC 29212 bacteria in presence of Zn^{2+}. The indicated *E. faecalis* isolates were incubated with HRGP (3 and 30 μM, respectively) in 10 mM Tris, pH 7.4 (black bars) or 10 mM Tris, pH 7.4, containing 50 μM Zn^{2+} (grey bars), and

the number of cfu was determined (n=2, mean values are presented). (B) Antibacterial activity of HRGP against *E. faecalis*, *S. aureus*, *E. coli and P. aeruginosa* in MES buffer at pH 5.5. The indicated *E. faecalis*, *S. aureus*, *E. coli*, and *P. aeruginosa* strains were incubated with HRGP (3 μM) in 10 mM Tris, pH 7.4 (black bars) or 10 mM MES, pH 5.5 (grey bars), and the number of cfu was determined. 100% survival indicates the number of bacteria in the respective buffer. Mean values are presented (n=2). **(**C) Antibacterial effects of HBP, histatin 5 and HRGP in diverse buffers. *E. faecalis* 2374 was incubated with 0.03-6 μM HBP (●), histatin 5 (○), or HRGP (▼) in 10 mM Tris, pH 7.4 with or without 50 μM Zn^{2+} or in 10 mM MES, pH 5.5. Samples were plated and the number of cfu determined. A representative experiment (of three) is shown. (D) Antibacterial effects of HBP, histatin 5 and HRGP in 0.15 M NaCl. *E. faecalis* 2374 was incubated with 6 μM HBP, histatin 5 or HRGP in 10 mM MES buffer, pH 5.5 containing 0.15 M NaCl, and the number of cfu were determined (n=6). When indicated, error bars represent SD.

Fig. 3. Negative staining and electron microscopy analysis of bacteria subjected to HRGP. Bacteria (*E. faecalis* 2374) were incubated in the absence of HRGP in 10 mM Tris, pH 7.4 (A), 10 mM Tris, pH 7.4 containing 50 μM Zn^{2+} (B), 10 mM MES, pH 5.5 (C), or 10 μM HRGP in 10 mM Tris, pH 7.4 without Zn^{2+} (D). These bacteria did not exhibit signs of membrane perturbations. In contrast, when bacteria were treated with 10 μM HRGP in 10 mM Tris, pH 7.4 containing 50 μM Zn^{2+} (E) or 10 mM MES, pH 5.5 (F), extensive membrane damage, blebbing and ejection of cytoplasmic components was observed (arrowheads). Examination of specimens at higher magnification showed intact plasma membranes for bacteria in 10 mM Tris, pH 7.4, containing 50 μM Zn^{2+} (G) or in 10 mM MES pH 5.5 (I). Upon treatment with 10 μM HRGP in 10 mM Tris, pH 7.4 containing 50 μM Zn^{2+} (H) or 10 mM MES, pH 5.5 (J) membrane integrity was severely disturbed and cytoplasm ejections

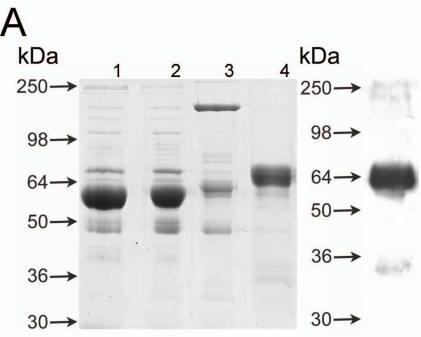
were frequently observed (arrowheads). (K). Bacteria treated with 10 μM LL-37 were used as a positive control for membrane damage. The scale bar in J corresponds to 250 nm and applies for G-J, whereas the bar in K corresponds to 1 μm and also applies for A-F.

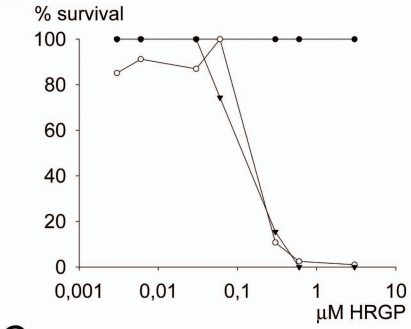
Fig. 4. Bacterial viability after incubation with HRGP. (A) Proportion of live and dead bacteria after exposure to HRGP. *E. faecalis* (1 x 10^7 bacteria) was incubated with 1 μM HRGP for two hours in 10 mM Tris, pH 7.4 containing 50 μM Zn^{2+} or 10 mM MES, pH 5.5. After incubation the proportion of live (impermeable) and dead (permeabilised) bacteria was determined using the LIVE/DEAD baclight kit. Identical buffers without HRGP were used as controls (labelled C). Significance was determined using Kruskall-Wallis one-way ANOVA analysis (*** $p<0.001$, n=6). (B) Illustration of a typical "pattern" obtained using 10 mM Tris, pH 7.4 containing 50 μM Zn^{2+}. Upper panels show bacteria incubated in buffer without the addition of HRGP, lower panels show bacteria incubated with HRGP. The left panels show Nomanski images. In fluorescence microscopy bacterial cells with compromised membranes fluoresce red (D) and those with intact membranes fluoresce green (L).

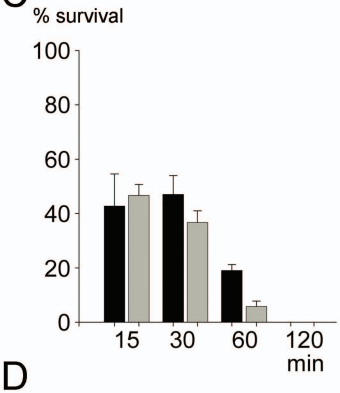
Fig. 5. Bacterial binding of histidine-rich glycoprotein is mediated via a heparin-binding region of the protein. (A) Binding of HRGP to bacteria. *E. faecalis* (1 x 10^5 bacteria) was incubated with HRGP (0.6 μM) in 10 mM Tris containing 50 μM Zn^{2+}, pH 7.4. For inhibition studies, heparin (50 μg/ml) was added. Samples were centrifuged and the pellet and supernatants were extracted and run on 8% SDS-PAGE. HRGP was detected by western and immunoblotting using polyclonal antibodies against GHH20. Purified HRGP was used as a positive control (labelled C). (B) Comparison of the antibacterial effect of rHRGP and the truncated version rHRGP1-240. *E. faecalis* (1 x 10^5 bacteria) was incubated with 0.6 μM

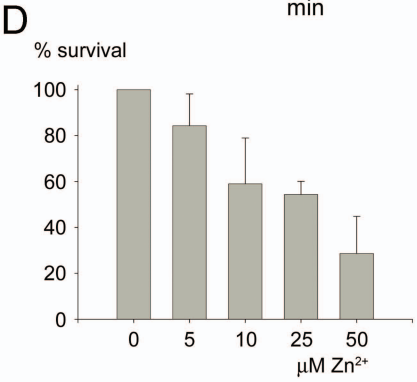
rHRGP or rHRGP1-240 in 10 mM Tris, pH 7.4 containing 50 μM Zn^{2+} or in 10 mM MES, pH 5.5. Samples were plated and the number of cfu was determined. Significance was determined using Kruskall-Wallis one-way ANOVA analysis (*** $p<0.001$, n=6) (C) Comparison of the amino acid sequences of human, mouse, rat, bovine, and rabbit HRGP. The region corresponding to the HRR (residues 330-389) in the human HRGP sequence is indicated by bold letters. A highly conserved region containing the prototypic GHHPH motif is boxed. (D) Purified HRGP and the GHH20 peptide binds to heparin. HRGP and the GHH20 peptide at the indicated concentrations were applied to nitrocellulose membranes followed by incubation with iodinated (^{125}I) heparin in 10 mM Tris, pH 7.4 or MES, pH 5.5. The presence of 50 μM Zn^{2+}, or buffer at pH 5.5 potentiated heparin-binding. (E) Binding of TAMRA-labelled GHH20 peptide to *E. faecalis* bacteria and inhibition by an excess of heparin. *E. faecalis* bacteria were incubated with TAMRA labelled GHH20 in 10 mM Tris buffer only (panel 1), buffer with 50 μM Zn^{2+}(panel 2) or the Zn^{2+} containing Tris buffer supplemented with heparin (50 μg/ml)(panel 3). The upper part shows Nomarski images, whereas the lower part show red fluorescence of peptide bound to bacteria. (F) Antibacterial activity of GHH20 against *E. faecalis* and *E. coli* bacteria. *E. faecalis* 2374 (left graph) was incubated with GHH20 peptide in 10 mM Tris pH 7.4 (●), 10 mM Tris, pH 7.4 containing 50 μM Zn^{2+} (○) or in 10 mM MES, pH 5.5 (▼). *E. coli* 80 (right graph) was only tested in 10 mM Tris pH 7.4 (●) and in 10 mM MES, pH 5.5 (▼). The bacteria were incubated with GHH20 peptide at the indicated concentrations and the number of cfu was determined. A representative experiment (of three) is shown. When indicated, error bars represent SD.

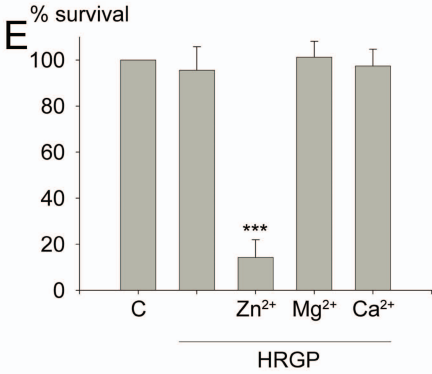
Figure 1

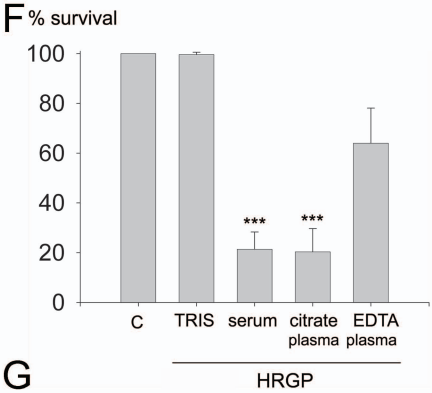

A
kDa
1
2
3
4
kDa
250
98
64
50
36
30
250
98
64
50
36
30


B
% survival
100
80
60
40
20
0
0,001
0,01
0,1
1
10
µM HRGP


C
% survival
100
80
60
40
20
0
15
30
60
120
min


D
% survival
100
80
60
40
20
0
0
5
10
25
50
µM Zn2+


E
% survival
100
80
60
40
20
0
***
C
Zn2+
Mg2+
Ca2+
HRGP


F
% survival
100
80
60
40
20
0
***
***
C
TRIS
serum
citrate plasma
EDTA plasma
HRGP

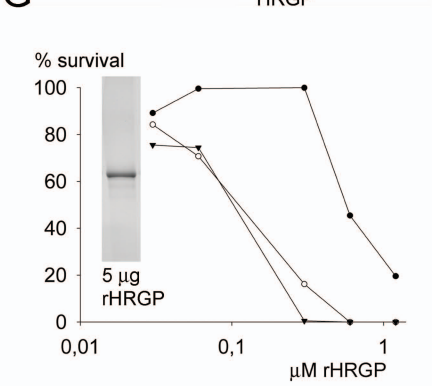
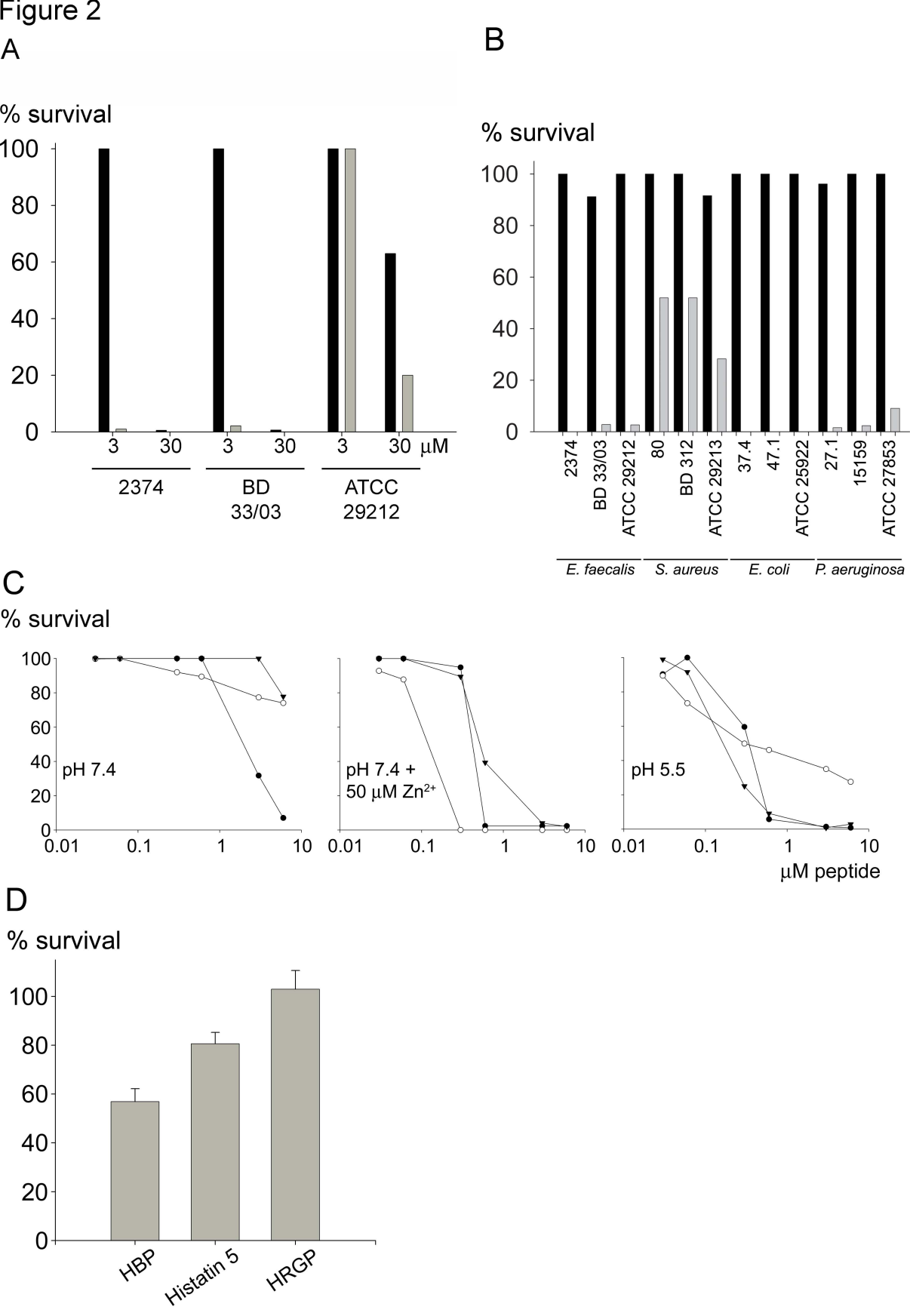

G
% survival
100
80
60
40
20
0
5 µg rHRGP
0,01
0,1
1
µM rHRGP

Figure 2


A
% survival
100
80
60
40
20
0
3
30
3
30
3
30 µM
2374
BD 33/03
ATCC 29212
B
% survival
100
80
60
40
20
0
2374
BD 33/03
ATCC 29212
80
BD 312
ATCC 29213
37.4
47.1
ATCC 25922
27.1
15159
ATCC 27853
E. faecalis
S. aureus
E. coli
P. aeruginosa
C
% survival
100
80
60
40
20
0
pH 7.4
pH 7.4 +
50 µM Zn2+
pH 5.5
0.01
0.1
1
10
µM peptide
D
% survival
100
80
60
40
20
0
HBP
Histatin 5
HRGP

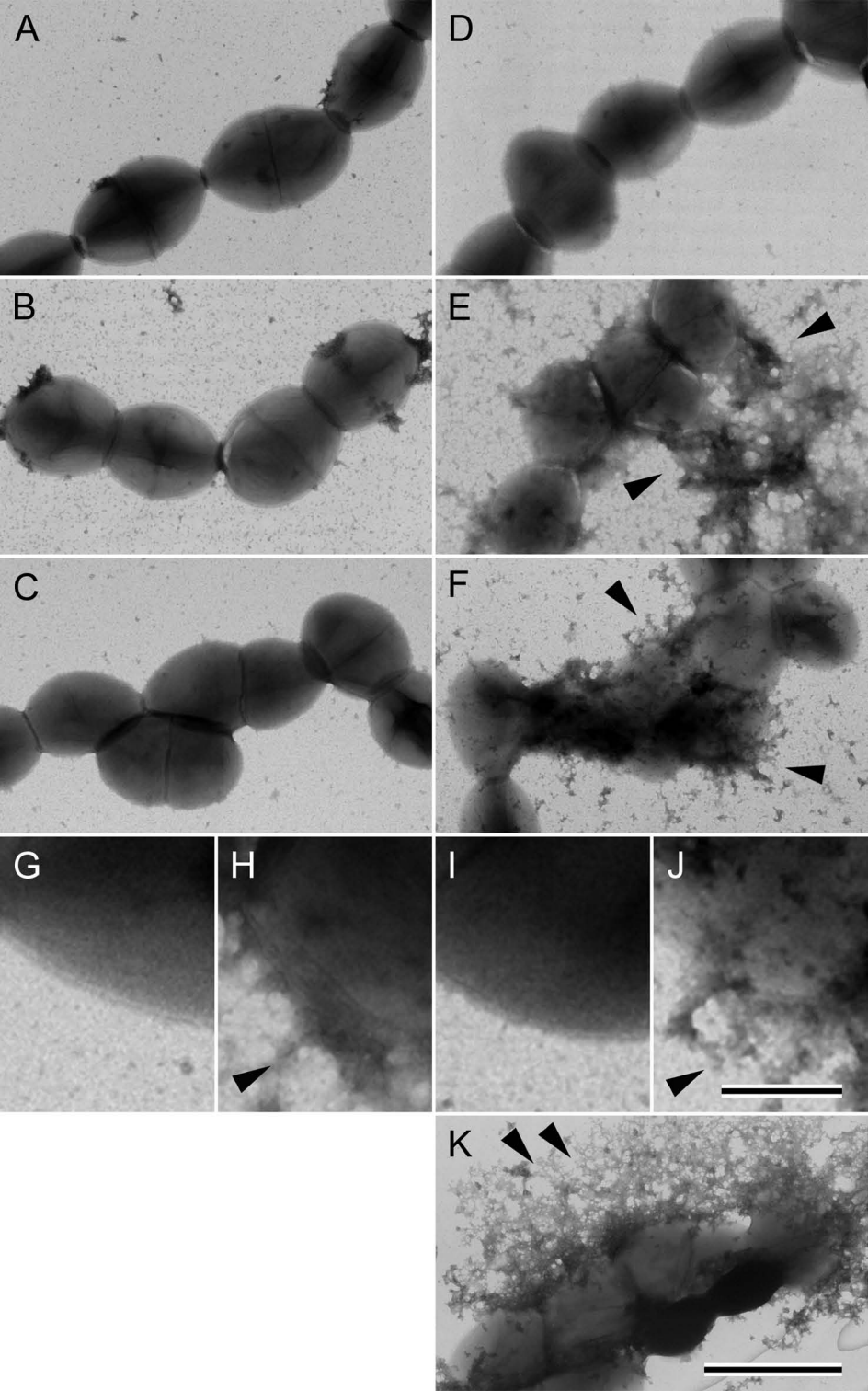
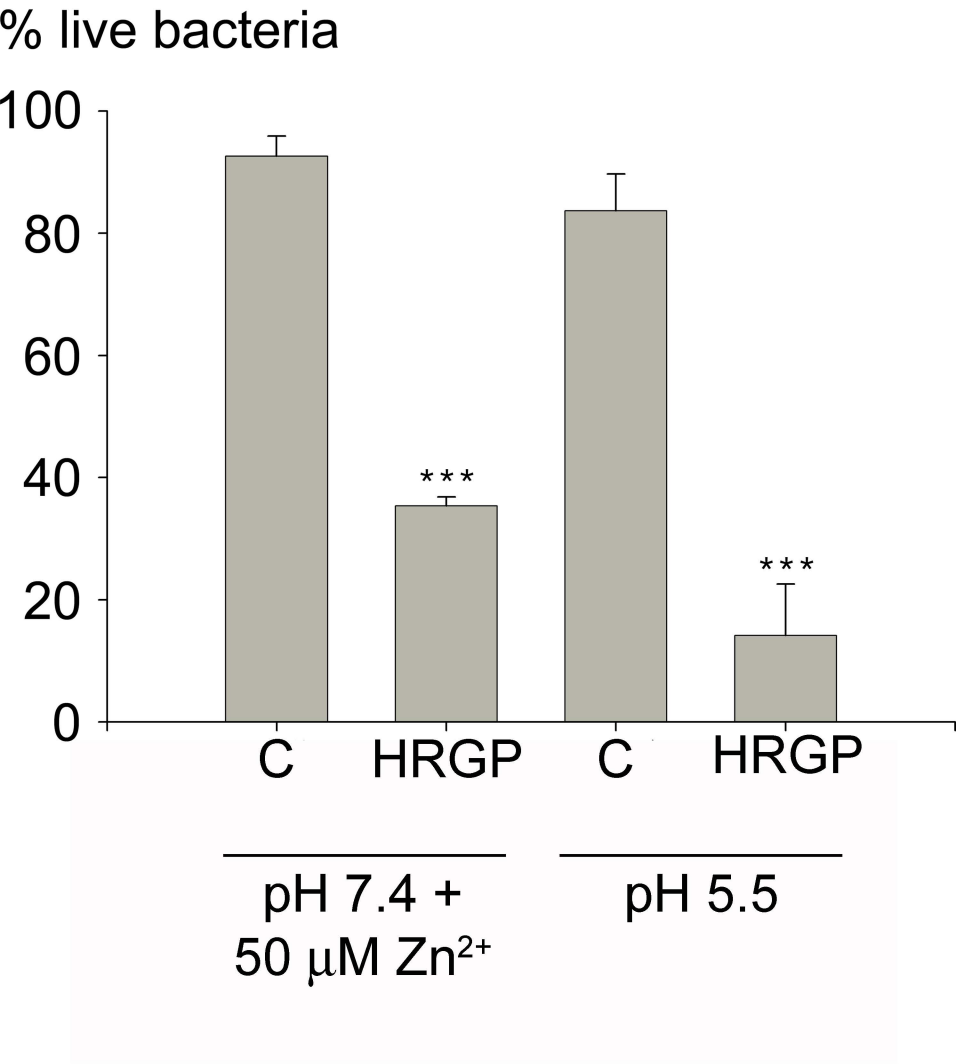

A
D
B
E
C
F
G
H
I
J
K

Figure 4

A


% live bacteria
100
80
60
40
20
0
***
***
C
HRGP
C
HRGP
pH 7.4 +
50 μM Zn2+
pH 5.5

B

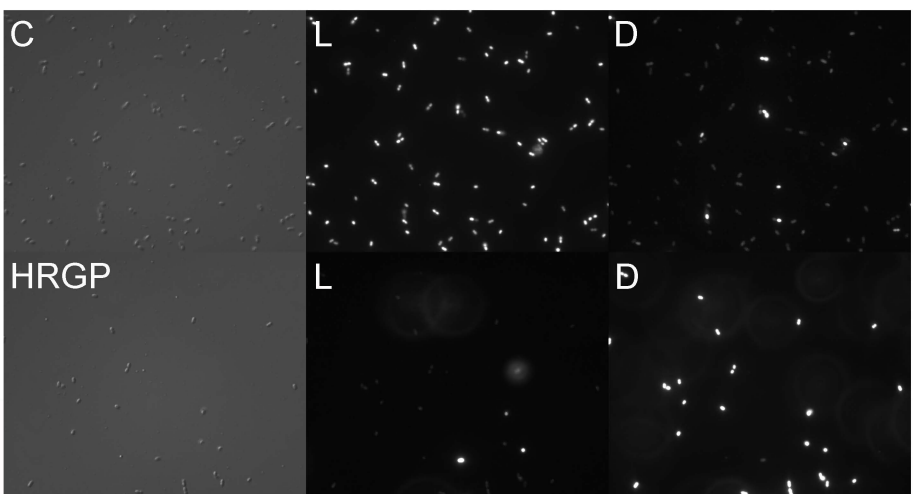
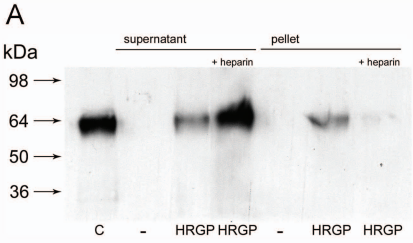
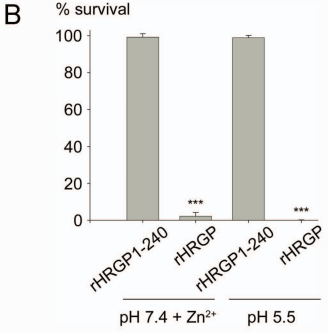

C
L
D
HRGP
L
D

Figure 5





C

```
HRG_HUMAN     SSD--LHP--HKHHSHE-----HGHHPHAHHPH----------EHDTHRQHPHGHHPHGHHPHGHHPHGHHPHGHHPHCHDF
Q6YK32_MOUSE  CNE---HP-CHGHRPHG-----HSHHPPGHHSHG-----HHPHGHHPHSHHSHGHHPPGHHPHGHHPHGHHPHGHHPHGHDF
Q99PS8_RAT    CNE---HP-CHGQHPHG-----HGQHPHGHHPHG-----QHPHGHHPHGQHPHGHHPHGQHPHGHHPHGHHPHGDHPHGHHPHGHDF
Q9BGU1_BOVIN  SDE--HHP--HGHHPHG-----HGHHPHGHHPHGHQPHGHHPHGHHPHGHHPHGHHPHGHHPHGHHPHGHHPYGHHPHGHHPHDNDF
HRG_RABIT     SVNIIHRPPPHGHHPHGPPPHGHGPPPHGHPPHGPPPR-HPPHGPPPHGHPPHGPPPHGHPPHGPPPHGHPPHGPPPHGHPPHGHGF
```