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Human Parotid Secretory Protein is a lipopolysaccharide-binding protein: identification of an anti-inflammatory peptide domain

Mahsa Abdolhosseini[a], **Julie B. Sotsky**[b], **Anuradha P. Shelar**[b], **Paul B.M. Joyce**[c], and **Sven-Ulrik Gorr**[a,b,d]

[a]Department of Diagnostic and Biological Sciences, University of Minnesota School of Dentistry, Minneapolis, Minnesota 55455, U.S.A

[b]Oral Health and Systemic Disease Research Group, Department of Periodontics, Endodontics and Dental Hygiene, University of Louisville School of Dentistry, Louisville Kentucky 40292, U.S.A

[c]Department of Chemistry and Biochemistry and the Centre for Structural and Functional Genomics, Concordia University, Montreal, Canada

Abstract

Parotid Secretory Protein (PSP) (C20orf70) is a salivary protein of unknown function. The protein belongs to the palate, lung and nasal epithelium clone (PLUNC) family of mucosal secretory proteins that are predicted to be structurally similar to lipid-binding and host defense proteins including bactericidal/permeability-increasing protein and lipopolysaccharide-binding protein. However, the PLUNC proteins exhibit significant sequence variation and different biological functions have been proposed for different family members. This study tested the functional implications of the proposed similarity of PSP to the acute phase protein Lipopolysaccharide-Binding Protein (LBP). PSP was identified in human saliva and was soluble in 75% ethanol, as shown for other PLUNC proteins. PSP binds lipopolysaccharide and can be eluted by non-ionic detergent, but not by urea or high salt. A synthetic PSP peptide, GL13NH2, which corresponds to a lipopolysaccharide-inhibiting peptide from LBP, inhibited the binding of lipopolysaccharide to both PSP and lipopolysaccharide-binding protein. Peptides from other regions of PSP and the control peptide polymyxin B showed no effect on the binding of PSP to lipopolysaccharide. GL13NH2 also inhibited lipopolysaccharide-stimulated secretion of tumor necrosis factor from macrophages. The other PSP peptides had no effect in this assay. PSP peptides had no or only minor effect on macrophage cell viability. These results indicate that PSP is a lipopolysaccharide-binding protein that is functionally related to LBP, as suggested by their predicted structural similarities.

Keywords

Saliva; endotoxin; SPLUNC2; C20orf70; antimicrobial peptide; lipopolysaccharide-binding protein

[d]Address correspondence to: Sven-Ulrik Gorr, Ph.D., University of Minnesota School of Dentistry 18-208 Moos Tower, 515 Delaware Street SE, Minneapolis MN 55455, Phone: 001-612-626-5728, FAX: 001-612-625-6948, sugorr@umn.edu.

INTRODUCTION

Human Parotid Secretory Protein (hPSP) (C20orf70) is a human salivary protein that is expressed in acinar and ductal cells of the parotid gland, submandibular gland and in gingival epithelial cells [1–4]. Human PSP belongs to the palate, lung and nasal epithelium clone (PLUNC) gene family located on chromosome 20q11 [5] and is related to similar proteins expressed in salivary glands of mouse [6], rat [7], hamster [8]; pig [9]; horse [10] and cattle (BSP30) [11]. A search of the NCBI data base revealed additional predicted PSP genes in dog, chimpanzee, orangutan, rhesus macaque, marmoset and giant panda.

The PLUNC family consists of mucosal secretory proteins that are predicted to contain one or two BPI superfamily domains (NCBI CDD cl00188), which are also found in bactericidal/permeability increasing protein (BPI), lipopolysaccharide (LPS)-binding protein (LBP), cholesteryl ester transport protein and phospholipid transport protein [5,12,13]. Despite this predicted structural similarity, the PLUNC proteins exhibit significant sequence variation and different biological functions have been proposed for different family members [14–20]. In particular, human PLUNC has been found to bind LPS [19] while bovine BSP30 does not exhibit LPS-binding activity [21]. Thus, it is not clear if the proposed BPI-like structure of the PLUNC proteins has implications for biological function.

In this report we have used previously designed PSP peptides to deduce a biological function for PSP. The PSP peptide design was based on the identification of LBP peptides that inhibit LPS binding to LBP and prevent LPS-induced secretion of TNFα from macrophages [22]. Since the PSP peptides exhibit similar activities [1], we tested whether PSP is also an LPS-binding protein. This study reports that PSP binds LPS and that binding is inhibited by the PSP peptide GL13NH2. This peptide also inhibits LPS binding to LBP and the LPS-stimulated secretion of TNF from macrophages. These findings suggest a biological function for PSP and validate the use of the proposed PSP structure for functional studies.

MATERIALS AND METHODS

Peptides and reagents

PSP peptides (Table 1) were synthesized by Peptides International, Louisville, Kentucky or the University of Minnesota peptide synthesis core. Polymyxin B and *Pseudomonas aeruginosa* LPS were from Sigma Chemical Co (St. Louis, MO). Monophosphoryl lipid A (MPLA) was from Invivogen (San Diego, CA). Control samples for the peptide experiments contained an equal volume of 0.01% acetic acid. Media and buffers were tested for LPS contamination by the limulus amebocyte lysate assay (Pyrogent Gel Clot LAL assay; Lonza, Walkersville, MD). An antiserum to human PSP was a kind gift from Dr. Thomas T. Wheeler, AgResearch, New Zealand. The antibody was validated by reaction with recombinant human PSP expressed in *E. coli* (not shown) or GH4C1 cells (Figure 3B).

Saliva samples

Saliva collection was approved by the Institutional Review Board of the University of Louisville (protocol 335.07). Whole saliva was collected on ice from healthy volunteers, using mechanical (chewing action) or citrus stimulation. Saliva was centrifuged 30 min at 3,000 × g and the resulting supernatant (saliva supernatant) stored at −20°C prior to use. In some experiments, the saliva supernatant was precipitated with three volumes of cold 95% ethanol and incubated 15 min at 4°C. The samples were centrifuged at 3,000 × g for 1 h and the ethanol supernatant fraction mixed with 2.5 volumes of ice-cold acetone. The samples were incubated at 4°C and centrifuged at 3,000 × g for 30 min. The pellet was resuspended

in PBS and stored at −20°C until use. One ml of this "saliva ethanol supernatant" corresponds to 10 ml "saliva supernatant".

Saliva degradation—Aliquots of saliva supernatant were incubated overnight at −20°C, 4°C, 21°C (room temperature) or 37°C. The samples were boiled in SDS-PAGE sample buffer and stored frozen until analysis.

Recombinant PSP—Human PSP was expressed in rat pituitary GH4C1 cells that were transfected with the plasmid pcDNA3 containing a wild-type human PSP cDNA under the control of the CMV promoter [2]. PSP expression was enhanced by treating the transfected cells with 5 mM sodium butyrate in DMEM [23]. Control media came from GH4C1 cells that were transfected with a plasmid containing the cDNA insert in reverse orientation, which does not allow PSP expression [2]. Secretion medium was collected after 24 hours and centrifuged 10 min at 1,000 × g to remove cells and cell debris before use.

LPS pull-down experiments

LPS-beads were prepared by coupling *P. aeruginosa* LPS (10 mg/ml) to CNBr-Sepharose 4 fast flow beads (GE Health Care) following the manufacturer's instructions. Saliva supernatant was diluted 1:6 in 10 mM sodium phosphate, pH 7.4. Five ml diluted supernatant or five ml GH4C1 secretion medium was mixed with a 500 μl slurry of LPS-beads overnight at 4°C. The beads were centrifuged (200 × g, 90 s) and washed with 3 × 0.5 or 1 ml PBS followed by elution in PBS supplemented with either 0.5 mM EDTA or 8 M urea or 1% Tween 20 or 1 M NaCl. The beads were centrifuged and the supernatants (eluate) were precipitated with 80% acetone and analyzed by SDS-PAGE and immunoblotting. Bound proteins were detected by boiling the eluted beads in SDS-PAGE sample buffer followed by SDS-PAGE and immunoblotting of the supernatant, as previously described [24].

For peptide inhibition experiments (Figure 1B–C), the beads (50 μl slurry) were incubated with 5 μl saliva supernatant or saliva ethanol supernatant and 100 μg/ml peptide. The volume was adjusted to 200 μl with 10 mM sodium phosphate pH 7.4 or PBS and the samples were incubated overnight at 4°C followed by washing in PBS. Bound proteins were detected by boiling the beads in SDS-PAGE sample buffer followed by SDS-PAGE and immunoblotting of the supernatant.

LBP-binding assay

The effect of PSP peptides on the binding of LPS to LPS-binding protein was quantitated by a commercial assay (Endoblock LBP ELISA; Hycult Biotech, Uden, The Netherlands). The assay was performed according to the manufacturer's protocol using 0–180 μg/ml of the PSP peptides or polymyxin B as inhibitors. Biotinylated LPS bound to LBP was reacted with streptavidin-peroxidase and detected colorimetrically.

Tumor necrosis factor-α (TNFα) secretion assay

RAW 264.7 cells were cultured in RAW medium overnight in 24-well plates as described [1]. LPS or MPLA (100 ng/ml) and 200 μg/ml peptide were pre-incubated in RAW medium for 1 h at 37°C prior to addition to the RAW cells. The cells were then incubated overnight at 37°C, 5% CO_2 after which the media were collected, chilled on ice and centrifuged 5 min at 21 000 × g. The supernatants were stored frozen or used directly for serial dilution and analysis by TNFα ELISA, following the manufacturer's instructions (eBioscience, San Diego, CA).

To test the effect of PSP peptides on the TNFα assay, TNFα ELISA standard curves were prepared in the presence of 100 μg/ml of GK7, GL13NH2 or buffer control (0.01% acetic acid). The PSP peptides did not affect the detection and quantitation of TNFα in this assay (not shown).

Viability assay

RAW 264.7 cells were cultured overnight in 24-well plates as described [1] and the medium was replaced with 0.5 ml fresh RAW medium [1] containing 200 μg/ml of the PSP peptides or a buffer control. After 24 h incubation, the cellular ATP content was quantitated using the Cell Titer Glo assay (Promega, Madison, WI).

Statistical analysis

Data were analyzed by ANOVA with multiple comparison post tests, as indicated in figure legends. $P<0.05$ was considered statistically significant.

RESULTS

Human PSP is expressed in salivary glands as a 249 amino acid, hydrophobic glycoprotein where leucine, isoleucine and valine constitute 34% of the amino acid residues. PSP is related to PLUNC and BSP30, which are soluble in 75% ethanol [25] and 63% isopropanol [21], respectively. To test whether PSP is also soluble in alcohol, saliva was ethanol precipitated and analyzed by SDS-PAGE and immunoblotting (Fig. 1). While the majority of saliva proteins, including amylase, where precipitated by 70% ethanol, PSP was largely recovered in the supernatant fraction after centrifugation of the precipitates (Fig. 1). Thus, alcohol solubility appears to be a general property of members of the PLUNC family.

Human PSP is typically identified as two bands upon SDS-PAGE, including an N-glycosylated and non-glycosylated form [2,26,3]. To test whether these molecular forms are susceptible to proteolytic degradation, whole saliva was incubated overnight at different temperatures. The ratio of the upper and lower PSP bands did not change, even at 37°C, suggesting that the two forms are not differentially processed by proteolysis after secretion (Figure 2). Based on this result, all further experiments were performed under conditions that do not cause PSP degradation.

To test whether PSP binds LPS, saliva samples were incubated with LPS-Sepharose beads and bound PSP detected by immunoblotting (Figure 3). PSP was eluted with 1% Tween 20 but not by 8 M urea or 1 M NaCl. The protein was partially eluted by 0.5 mM EDTA (Figure 3A). To validate this binding, PSP was expressed in GH4C1 cells and the secretion medium used for LPS-pull down experiments. The bound protein was eluted with EDTA and detected by immunoblotting (Figure 3B). No binding was detected in medium from control cells that do not express PSP. In addition, PSP did not bind to control beads that did not contain LPS (data not shown). These results suggest that PSP mainly binds LPS by hydrophobic interactions. The finding that both PSP bands in saliva bound to LPS, suggest that N-glycosylation is not required for PSP binding [3].

We have previously designed anti-LPS [1] and bacteria agglutinating [27] peptides based on the predicted structure of PSP. To determine whether these peptides disrupt the LPS-binding activity of PSP, we performed PSP pull-down assays in the presence of each peptide (Figure 4a). The agglutinating peptide, GL13NH2, inhibited the binding of PSP to LPS beads, while the peptides GK7 and KL11 had no effect. LPS binding was not inhibited by the LPS-binding peptide polymyxin B. To further test the inhibition by GL13NH2, the peptide was modified by adding or removing an amide: In one modification the Asp residue in position 11 was replaced with Asn (GL13D/N) to add an amide. In another modification the C-

terminal amide was removed (GL13OH) (Table 1). Neither modification affected the peptide's ability to inhibit PSP binding to LPS (Figure 4b).

To test whether the GL13 sequence acts as the LPS binding site in PSP, we attempted to generate a PSP mutant where the Leu residues of the GL13 domain were replaced by Ser residues. However, as noted with another PSP mutant [2], the resulting protein was not expressed in detectable levels in mammalian cells, presumably due to incorrect folding and intracellular degradation (data not shown).

To determine whether GL13NH2 specifically inhibits LPS-binding to PSP, the peptide was also tested in an LPS-binding protein assay. GL13NH2 blocked the binding of LPS to LPS-Binding Protein in a dose-dependent manner. The LPS-binding peptide polymyxin B also blocked binding while GK7NH2 had no effect under these conditions (Figure 5).

The finding that GL13NH2 can block the binding of LPS to PSP and LBP, prompted us to test whether the peptide also blocks the action of LPS in cellular activation. GL13NH2 inhibited the secretion of TNFα from LPS-activated RAW 264.7 macrophage cells (Figure 6a). The PSP peptides did not stimulate TNFα secretion in the absence of LPS, suggesting that they do not exhibit inflammatory activity (not shown).

To test whether the effect of GL13NH2 was specific for LPS, RAW 264.7 macrophage cells were stimulated with the LPS-analog MPLA, an immune adjuvant that weakly stimulates toll-like receptor 4 [28]. MPLA-stimulated TNFα secretion was only about 10% of that of the LPS-stimulated secretion and was completely inhibited by GL13NH2. In contrast, while polymyxin B inhibited TNFα secretion by about 25%, GK7 and KL11 had no effect (Figure 6b). The reduced TNFα secretion in peptide-treated cells was not due to peptide toxicity, since GK7, KL11 and GL13NH2 had no effect on cell viability. In contrast, polymyxin B reduced cell viability by about 75% (Figure 7). Similarly, GL13NH2 did not affect proliferation of RAW cells over 5 days (data not shown).

DISCUSSION

Based on structural predictions, it has been proposed that PSP plays a role in host-defense, including the recognition of LPS [20]. However, conflicting LPS-binding results have been reported for the related proteins, PLUNC and BSP30 [21,19,25]. In this study, we determined that PSP binds to LPS, likely through hydrophobic interactions. Thus, the results for PSP are consistent with those for PLUNC but differ from those reported for BSP30. A possible difference in these experiments is that free PSP or PLUNC was bound to immobilized LPS, whereas immobilized BSP30 was reacted with free LPS. Thus, it is possible that the LPS binding site of BSP30 was either bound to or blocked by the affinity matrix or that the conformation of the bound BSP30 differed enough from that of the free form that it was unable to bind to LPS. Both the glycosylated and unglycosylated forms of PSP [3] bound LPS and binding was inhibited by the GL13 peptides. Thus, although the N-glycosylation sites (Asn residues marked with asterisks) are located near the GL13 sequence (underlined) in PSP [GLN*LSFPVTAN*VTVAGPIIGQIINLKASLDLL] [26] it appears that glycosylation does not affect the binding of PSP to LPS.

The binding of LPS to PSP was inhibited by GL13 peptides but not by other PSP peptides or polymyxin B. This finding suggests that the GL13 sequence is part of the LPS binding site in PSP, although this could not be directly tested due to the lack of expression of the appropriate PSP mutant. By design, the location of GL13NH2 in the predicted structure of PSP [1] is similar to the location of an anti-LPS peptide in the structure of LBP [22] and GL13NH2 inhibited the binding of LPS to LBP. This finding validates the use of the predicted structure of PSP for functional studies.

GL13NH2 inhibited secretion of TNFα after stimulation of macrophages with both LPS and MPLA, suggesting that the peptide reacted with the lipid A portion of LPS. The GK7 peptide did not inhibit TNFα secretion in these overnight experiments performed in the presence of 10% serum, in contrast to earlier experiments performed over 6h in DMEM [1], Thus, only the longer GL13 peptides appear to remain active under longer-term cell culture conditions. Consistent with this, GL13 peptides, but not the GK7 peptide, were able to inhibit LPS binding to PSP.

One rationale for using endogenous host-defense proteins rather than peptides from other species for the design of anti-inflammatory peptides is the potential for lower host toxicity. Indeed, GL13NH2 has shown little toxicity to mammalian cells in this and a previous report [27]. These findings provide hope that the PSP peptide may provide effective anti-inflammatory action with lower host toxicity than currently available anti-inflammatory peptides [29].

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Abbreviations

BPI	bactericidal/permeability-increasing protein
LPS	lipopolysaccharide
LBP	lipopolysaccharide-binding protein
MPLA	monophosphoryl lipid A
PLUNC	Palate, lung nasal epithelium clone
PMX	polymyxin B
PSP	- Parotid Secretory Protein
TNF*α*	tumor necrosis factor-α

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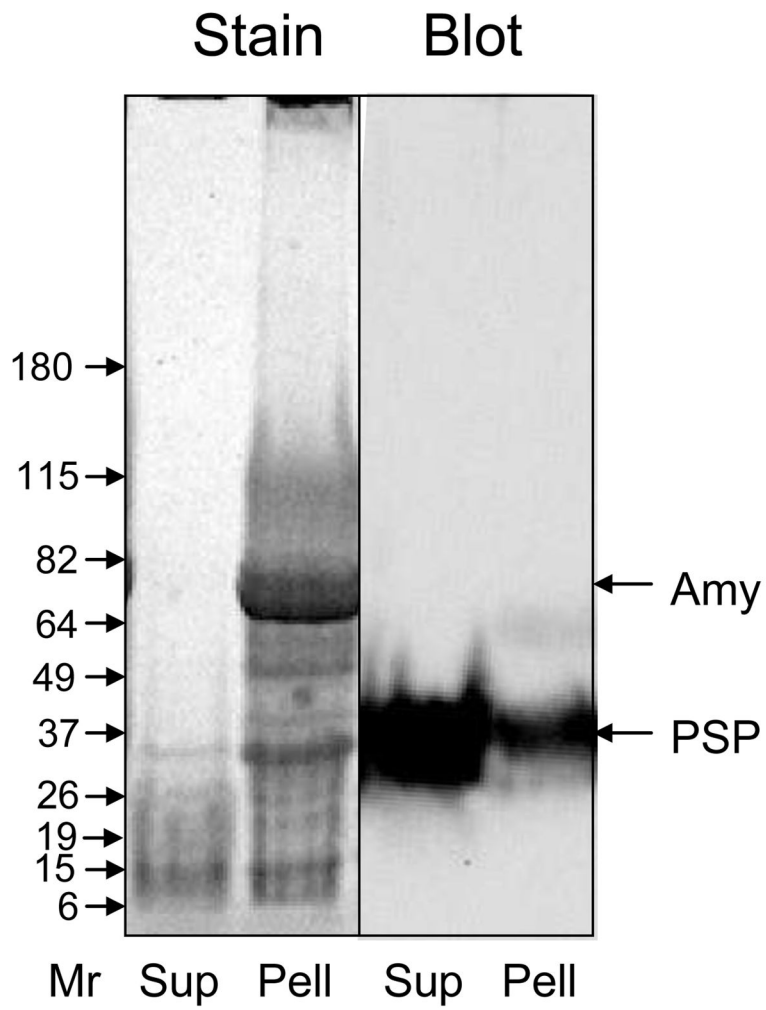

Figure 1. Ethanol precipitation

Saliva was precipitated with 70% ethanol and the supernatant (Sup) and pellet (Pell) fractions analyzed by SDS-PAGE followed by staining with Gelcode Blue (**Stain**) or immunoblotting with an antiserum to PSP (**Blot**). The positions of molecular mass markers are shown for the stained gel and the positions of amylase (Amy) and PSP (PSP) are shown.

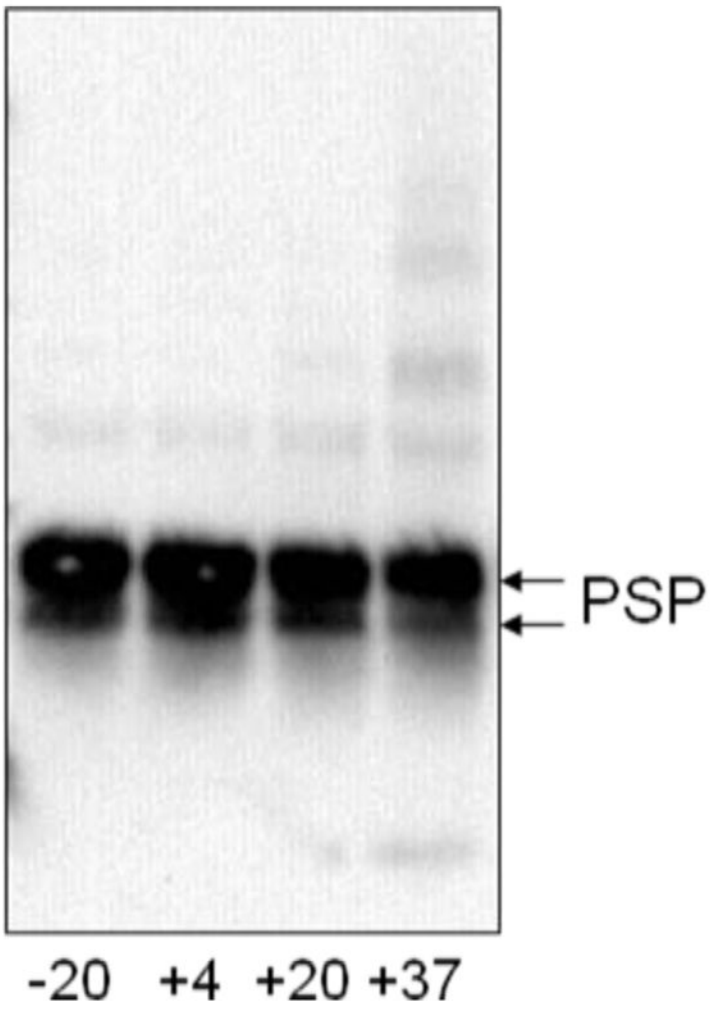

Figure 2. Stability of PSP in vitro

Saliva samples were incubated overnight at the temperatures indicated and then analyzed by immunoblotting for PSP. The positions of the two PSP bands are indicated by the arrows.

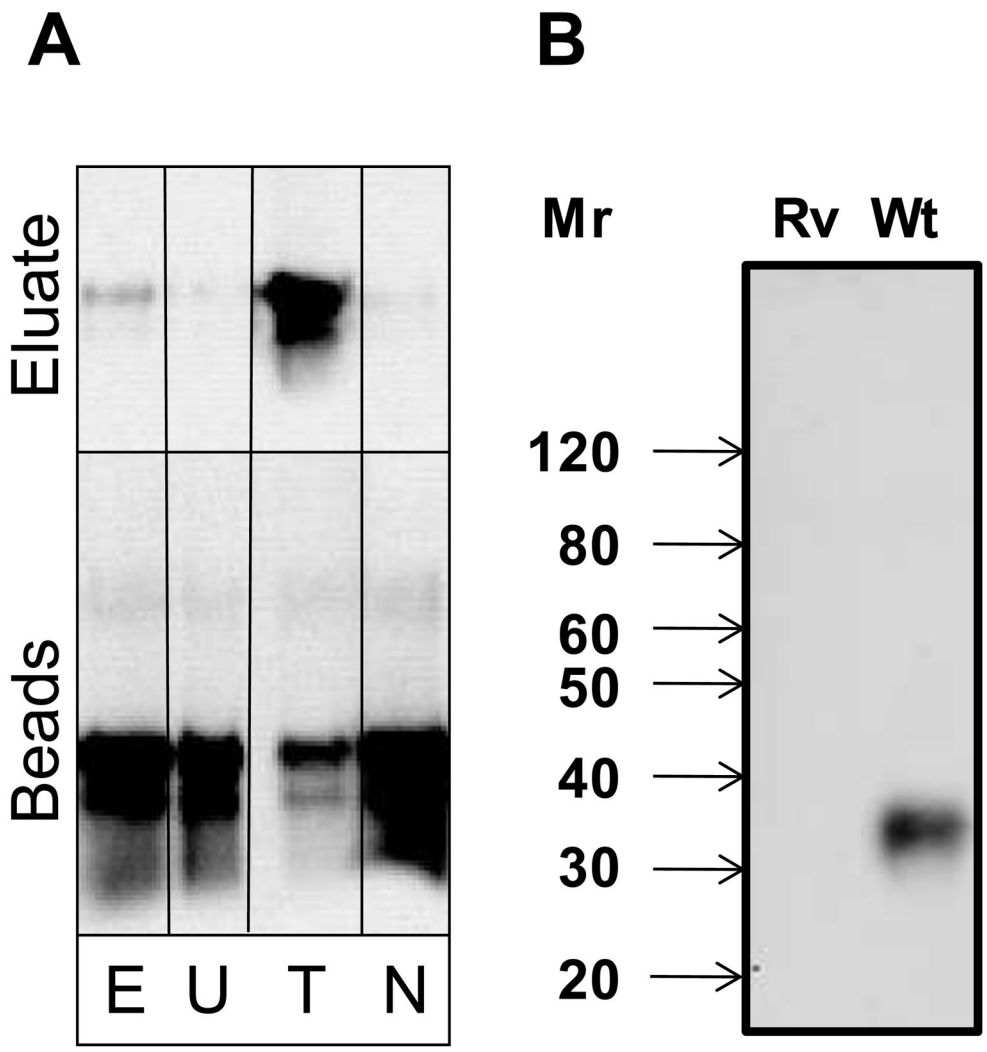

Figure 3. LPS binding of PSP

A: Saliva supernatant samples were incubated with LPS-Sepharose beads. The beads were washed and eluted with EDTA (E), Urea (U), Tween 20 (T) or NaCl (N) (Eluate). The eluted beads were then boiled in SDS-PAGE buffer to remove remaining bound PSP (Beads). PSP was detected by immunoblotting.

B: Wild-type PSP (Wt) or a reverse cDNA control (Rv) were expressed in GH4C1 cells and the culture media were incubated with LPS-Sepharose beads. Bound protein was eluted with EDTA and analyzed by immunoblotting. The position and size (kD) of relative molecular mass markers (Mr) are indicated by arrows.

Figure 4A

Figure 4B

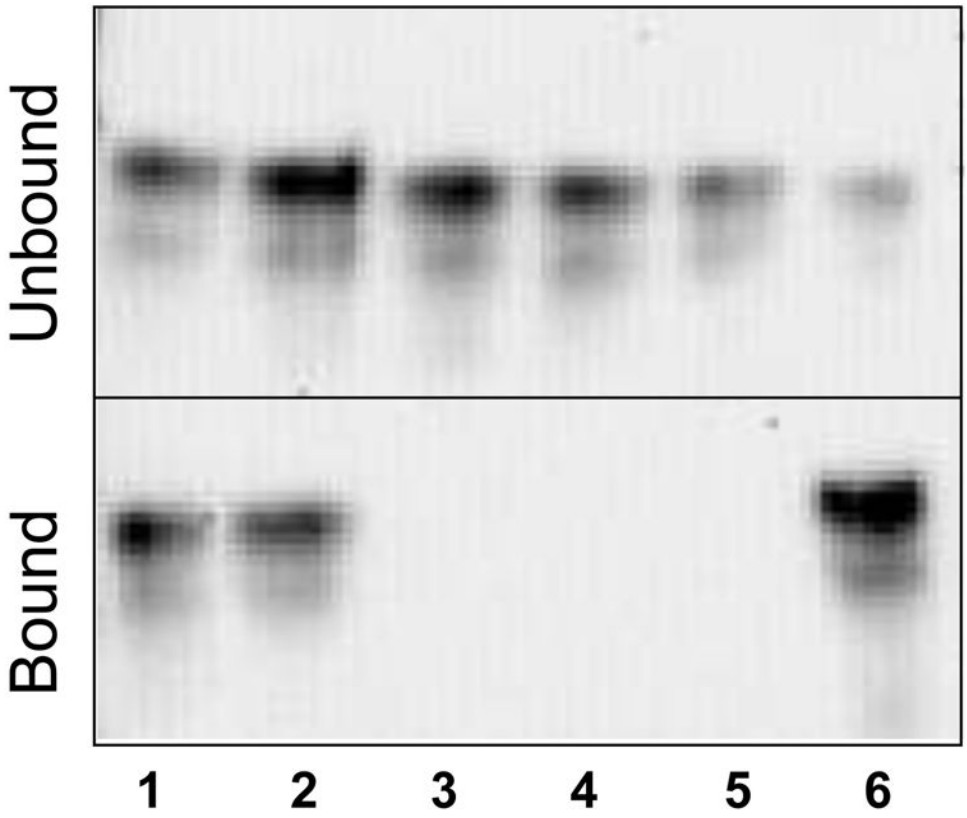

Figure 4.
Effect of PSP peptides on LPS binding.
Figure 4a: LPS-beads were incubated with 'saliva ethanol supernatant' in the absence (Blk) or presence of the PSP peptides GK7, GL13NH2 or KL11 as indicated. The beads were washed and bound PSP was analyzed by immunoblotting.
Figure 4b: LPS-beads were incubated with saliva supernatant in the absence of peptide (1), in the presence of GK7 (2), GL13NH2 (3), GL13OH (4), GL13DN (5) or polymyxin B (6). The unbound and bound fractions were analyzed by immunoblotting for PSP.

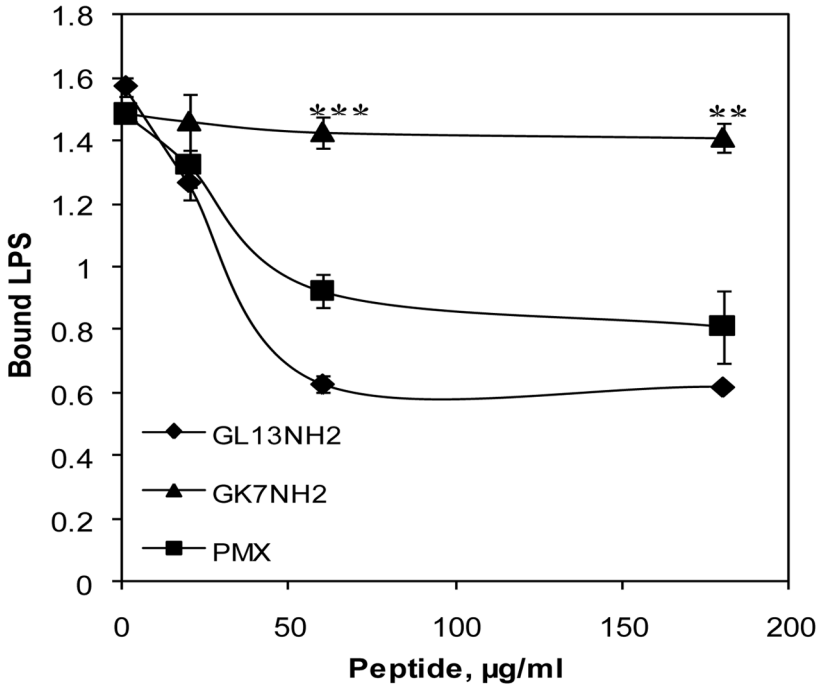

Figure 5. LPS binding to LBP

Biotinylated LPS was incubated with immobilized LBP in the presence of 0, 20, 60 or 180 µg/ml of GK7NH2 (triangles), GL13NH2 (diamonds) or polymyxin B (PMX) (squares). Bound LPS was detected spectrophotometrically and expressed as the absorption at 450 nm. Data from three independent experiments are expressed as mean ± SD, N=3 and analyzed by two-way ANOVA with Bonferroni multiple comparison post-tests. ***) GK7NH2 is different from the other peptides, $P<0.0001$.

Figure 6A

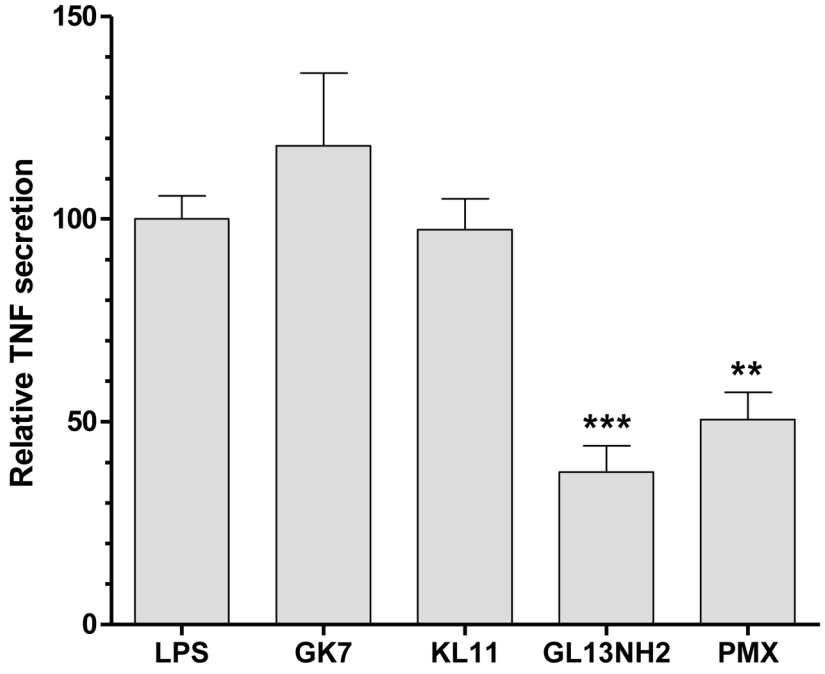

Figure 6B

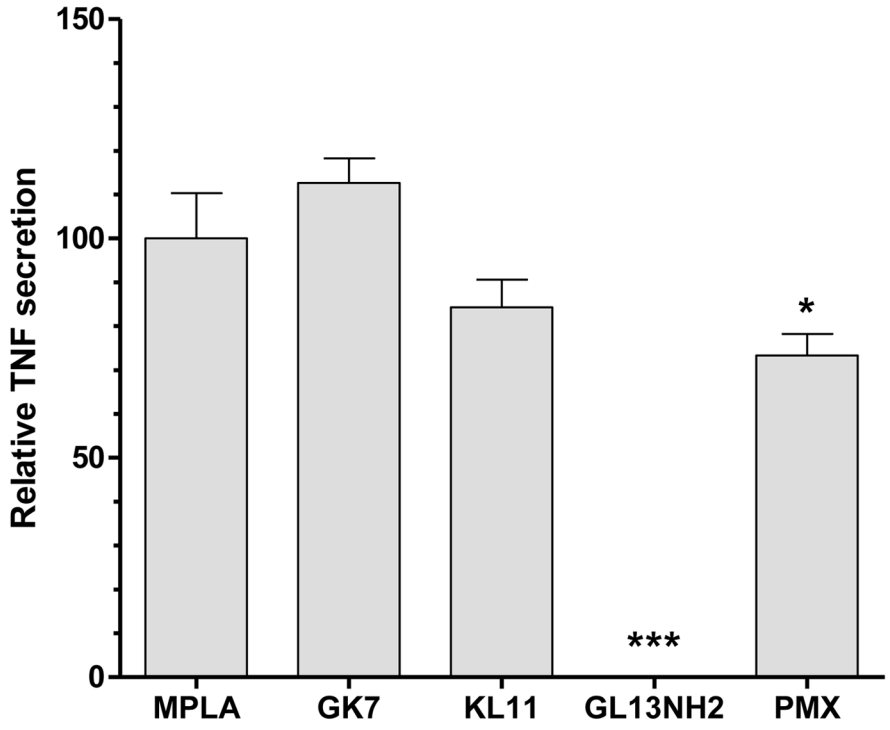

Figure 6.

a: LPS-stimulated TNFα secretion. RAW 264.7 cells were stimulated with 100 ng/ml *P. aeruginosa* LPS without added peptide (LPS) or in the presence of peptide GK7, KL11, GL13NH2 or polymyxin B (PMX) and incubated overnight. Secreted TNFα was quantitated by ELISA and expressed relative to LPS-stimulated secretion in the absence of peptide. Data from 2–5 independent experiments are expressed as mean ± S.E.M., N=6–21. The 100% value corresponds to 69 ng TNFα/ml. Data were analyzed by ANOVA and Dunnett's multiple comparison post-test. **) P<0.01, ***) P<0.001 compared to LPS alone.

b: MPLA-stimulated TNFα secretion. RAW 264.7 cells were stimulated with 100 ng/ml MPLA in the presence of peptide GK7, KL11, GL13NH2, or polymyxin B (PMX) or without added peptide (MPLA) and incubated overnight. Secreted TNFα was quantitated by ELISA and expressed relative to MPLA-stimulated secretion in the absence of peptide. The data shown are from a single experiment with three samples (N=3) and shown as mean ± SD. The 100% value corresponds to 4 ng TNFα/ml. Qualitatively similar results were obtained in two more independent experiments. Data were analyzed by ANOVA and Dunnett's multiple comparison post-test. *) P<0.05, ***) P<0.001 compared to MPLA alone.

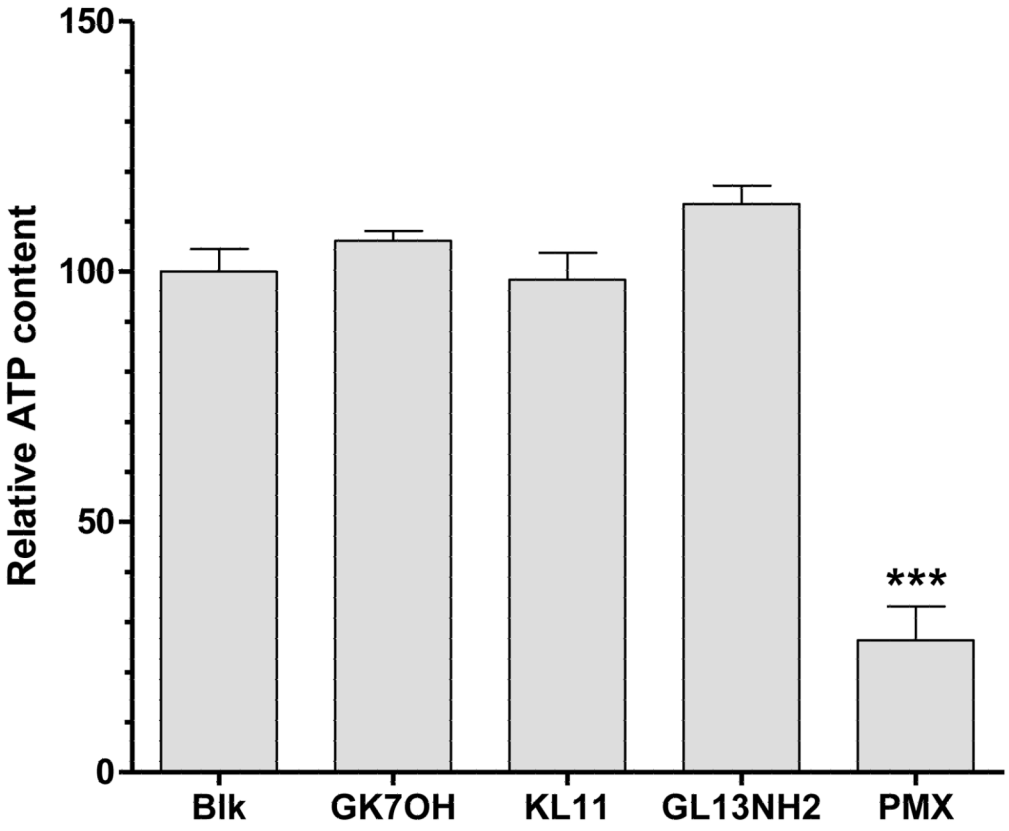

Figure 7. Raw cell viability

RAW 264.7 cells were cultured overnight in the absence of peptide (Blk) or in the presence of 200 μg/ml of GK7OH, KL11, GL13NH2 or polymyxin B (PMX). The cellular ATP content was quantitated and expressed relative to the blank (no peptide added) for each experiment. Data from two independent experiments are expressed as mean ± S.E.M., N=16. Data were analyzed by ANOVA and Dunnett's multiple comparison post-test. ***) $P<0.001$ compared to blank.

Table 1

Sequences of PSP peptides

The table lists the PSP peptides, their sequences and locations in the full-length PSP sequence. Peptide sequences ending in –OH contain a C-terminal free carboxylate; sequences ending in -NH2 contain a C-terminal amide. Amino acid substitutions are highlighted in bold and underlined. The LBP peptide H-14 [30] is shown for comparison

Peptide	Sequence	PSP location
GK7OH	GQIINLK-OH	141–147
GK7NH2	GQIINLK-NH2	141–147
KL11OH	KLLNNVISKLL-OH	83–93
GL13NH2	GQIINLKASLDLL-NH2	141–153
GL13OH	GQIINLKASLDLL-OH	141–153
GL13D/N	GQIINLKASL**N**LL-NH2	141–153
LBP H-14	CRWKVRKSFFKLQCG	LBP 90–101