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(54) Title: POLYPEPTIDES

(57) Abstract: The invention relates to polypeptides, encoded by nucleic acid which maps to human chromosome 20q11, which have antibacterial activity for use as a therapeutic agent. In addition it relates to the use of such polypeptides in the manufacture of a medicament for the treatment of bacterial infections in particular respiratory infections.



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Polypeptides

This invention relates to polypeptides which have anti-bacterial activity and compositions comprising said polypeptides.

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Animals are exposed to millions of potential pathogens daily through contact, ingestion and inhalation. These microbial organisms cause a number of fatal or debilitating diseases that affect many millions of people around the world, particularly in immunocompromised individuals. Currently, methods to control microbial organisms include the use of antimicrobial agents (antibiotics) and disinfectants. These have proved to be problematic since exposure to these agents places a significant selection pressure resulting in the creation of resistant microbes which can avoid the effects of the antimicrobial agent(s). For example, recently it has been discovered that microbial organisms have become resistant to the chlorophenol compound, triclosan, an agent added to many disinfectants used in households and industrial environments.

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An arguably greater problem is the evolution of antibiotic-resistant strains of a number of significant pathogenic microbes. With the emergence of antibiotic-resistance microbes, anti-pathogenic agents are an area of intense pharmaceutical research and development.

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The early recognition of bacterial products is critical for our survival. The presentation of pathogenic products to our innate immune system is complex but has been somewhat characterised for bacterial lipopolysaccharide (LPS) or endotoxin. The LPS constituents of bacterial cell walls, such as LPS of gram-negative bacteria, lipoteichoic acids of gram-positive bacteria and lipoarabinomannase of mycobacteria have profound clinical effects. The immune response to this bacterial invasion includes the activation of neutrophils and macrophages to release potent inflammatory mediators and destroy the invading bacteria.

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Several therapeutic compounds have been developed to inhibit the toxic effects of LPS/endotoxin, including antibacterial LPS-binding agents and anti-LPS antibodies, although these approaches have often been met with a number of limitations. For

example, Polymyxin, a basic polypeptide antibiotic that binds to Lipid A, the most toxic and biologically active component of endotoxin, inhibits endotoxin-mediated activation of neutrophil granule release *in vitro* and is a potential therapeutic agent for Gram-negative infections. However, in some instances Polymyxin B has shown to induce systemic toxicity and so this antibiotic is limited, generally, to topical use.

The administration of antibodies that bind endotoxin has had some success. For example, treatment of patients with Gram-negative bacteremia and shock with hyperimmune human antisera against *E.coli* J5 reduced mortality by 50% (Ziegler *et al.*, N. Engl. J. Med. 307: 1225, 1982). In comparison, attempts to treat Gram-negative sepsis by administration of anti-LPS monoclonal antibodies did not prove to be quite so successful (Ziegler *et al.*, N. Engl. J. Med. 324: 429, 1991; Greenman *et al.*, N. Engl. J. Med. 325: 279, 1991 and Baumgartner *et al.*, Infect. Dis. Clin. North. A. 5:915, 1991).

Another approach has been to prevent the recognition of LPS by LPS receptor complexes on the surface of macrophages. These cells require that the LPS must be bound to a secreted receptor protein that acts as a presentation molecule (Fenton & Golenbock, J. Leuko. Biol. 64: 25-32, 1998; Martin T.R, Am. J. Respir. Cell. Biol. 23:128-32, 2000). A well established family of four structurally homologous proteins involved in human defense against bacteria has been defined. These are the bactericidal/permeability-increasing protein (BPI), the LPS binding protein (LBP), the cholesteryl ester transfer protein (CETP) and the phospholipid transfer protein (PLTP). The two structurally related and soluble LPS-binding proteins, BPI and LBP have been identified as being critical and antagonistic roles in the physiological response to LPS. The soluble, high affinity LPS receptor, LBP, is found in serum where it binds LPS via interaction with the Lipid A moiety, thereby enhancing the cellular response to LPS (Tobias *et al.*, J. Exp. Med. 164: 777-793, 1986). LBP-LPS complexes stimulate monocyte activation through interaction with the CD14 receptor on the surface of monocytes, resulting in the production of proinflammatory cytokines such as IL-1 and TNF (Triantafyllou *et al.*, Trends. Immunol. 23:301, 2002). LBP can therefore be considered as a transfer protein in LPS-mediated stimulation of cytokine release.

In contrast to the action of LBP, BPI (also referred to as CAP57 or BP) binds and neutralises LPS, thereby preventing inflammatory cell activation and thus reducing the innate immune response (Shafer *et al.*, Infect. Immun. 45: 29, 1984 and Hovde *et al.*, Infect. Immun. 54: 142, 1986). In addition to its role in neutralizing LPS, BPI has also been shown to have a direct bactericidal action, by virtue of its interaction with the Lipid A moiety of LPS in the bacterial cell wall. This interaction disrupts the structure of LPS and the cell wall, increases bacterial membrane permeability, resulting in cell death (Weiss *et al.*, J. Biol. Chem. 253: 2664-2672, 1978 and Weiss *et al.*, Infect. Immun. 38: 1149, 1982). The identification of peptides derived from BPI and LBP as antibacterial therapeutics has been described in US 6,153,730 and US 6,162,788.

We describe the identification of a family of nine human proteins designated as PLUNCs (**p**alate **l**ung and **n**asal epithelium clone). The PLUNC proteins are encoded by adjacent genes in a *ca.* 300kB region on chromosome 20q11. Members of the PLUNC family fall into two groups based on their size. The proteins of smaller size are designated as SPLUNCs, for 'short' PLUNCs, comprising SPLUNC1 (256 aa), SPLUNC2 (249aa), SPLUNC3 (253aa or 210aa) and SPLUNC7 (229aa). The other group of larger proteins are designated as LPLUNCs, for 'long' PLUNCs, comprising LPUNC1 (484aa), LPLUNC2 (458aa), LPLUNC3 (463aa), LPLUNC4 (614aa) and LPLUNC6 (453aa). All of these proteins contain putative signal peptides at the N-terminus.

The pairwise amino acid identities between members of the PLUNC and BPI families are relatively low, ranging from 13-22%, but in terms of structural homology, the seven members of the PLUNC family have a predicted fold similar to that found in BPI. Structurally BPI comprises two homologous domains that dock onto each other via a central beta sheet, with the backbones of the two domains overlaying within 3 Angstrom. This two-domain structure of the BPI accounts for the division of the PLUNC family into short and long proteins, these groups containing either one or both of BPI's domains, respectively. The C-terminal domains of the SPLUNCs and LPLUNCs appear to demonstrate structural homology with BPI. However, whilst there does appear to be an indication of some structural homology between the PLUNC and BPI families, some fundamental differences are also indicated.

Whereas the BPI family are predicted to share a very closely similar secondary structure throughout both domains, in the PLUNC family, the N-terminal domain demonstrates a much greater variability, and this may have implications for specificity
5 of ligand binding.

Furthermore, three of the PLUNC proteins contain glycine-leucine rich sequences, which do not appear to conform to any well documented motif. In LPLUNC3, the sequence is **GLLGSGLLGGGGLLGHGG** (hydrophobic residues are in bold), in
10 LPLUNC4 the clearest sequence is **GGLLGGGGLLGDGGLLGGGGVLGVLGEGG**, whereas in SPLUNC1 there are two shorter sequences, **GLLSGLLG** and **GGGTSGGLLGGLL**G. The glycine linkers could provide these sequences with a large degree of conformational flexibility, enabling the hydrophobic residues to form a highly adaptable binding
15 surface. In respect to conserved residues, all of the PLUNC proteins conserve two cysteines in the putative LPS/lipid binding, N-terminal domain of the protein.

In respect to the expression patterns of these proteins, SPLUNC1 is most predominantly expressed in the nose, salivary gland and upper respiratory tract.
20 SPLUNC2 and LPLUNC1-4 exhibit a similar, although not identical expression pattern in tissues of these regions.

The fact that PLUNC genes are not only expressed in overlapping tissues but also found in a continuous locus on chromosome 20q11.2 suggests that common
25 regulatory regions may be required for targeting appropriate expression of these genes *in vivo*.

Therefore the PLUNC family of proteins appear to form a self contained family that is distinct from the BPI family, characterised by its co-localization within the genome
30 and expression in a number of overlapping locations, with a N-terminal diversity possibly conferring a high level of diversity in the proteins bactericidal response to micro-organisms.

According to an aspect of the invention there is provided a polypeptide wherein said polypeptide is selected from the group consisting of:

- 5 i) a polypeptide, or part thereof, encoded by a nucleic acid molecule which comprises a nucleic acid sequence which maps to a 300Kb region of human chromosome 20q11;
 - ii) a polypeptide encoded by a nucleic acid molecule which hybridizes to the nucleic acid molecule of (i) and which has anti-bacterial activity; and
 - 10 iii) a polypeptide encoded by a nucleic acid molecule which is degenerate as a result of the genetic code to the sequence identified in (i) and (ii)
- for use as a therapeutic agent.

In a preferred embodiment said nucleic acid hybridises under stringent hybridisation conditions.

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The term "stringent conditions" as used herein refers to parameters with which the art is familiar. Nucleic acid hybridization parameters may be found in references which compile such methods, e.g. *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. More specifically, stringent conditions, as used herein, refers, for example, to hybridization at 65°C in hybridization buffer (3.5 x SSC, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% Bovine Serum Albumin, 2.5mM NaH₂PO₄(pH7), 0.5% SDS, 2mM EDTA). SSC is 0.15M sodium chloride/0.15M sodium citrate, pH7; SDS is sodium dodecyl sulphate; and EDTA is ethylenediaminetetracetic acid. After hybridization, the membrane upon which the DNA is transferred is washed at 2 x SSC at room temperature and then at 0.1 - 0.5 x SSC/0.1 x SDS at temperatures up to 68°C.

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30 In a further preferred embodiment of the invention said polypeptide comprises an amino acid sequence selected from the group consisting of the sequences presented in Figure 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11 wherein said sequences are modified by addition, deletion or substitution of at least one amino acid residue.

Preferably said polypeptide consists of amino acid sequences as represented by Figures 1-11.

- 5 In a further preferred embodiment of the invention said polypeptide consists of a fragment of the amino acid sequences as presented in Figures 1-11.

In a further preferred embodiment of the invention said polypeptide comprises the amino-terminal domains of the sequences presented in Figures 1-4.

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In a preferred embodiment of the invention said domain consists of the amino acid sequence +1 to about +266 of the sequence presented in Figure 1, or part thereof. Preferably said polypeptide consists of sequences +31 to +67; +112 to +140; or +161 to +204 of the sequence presented in Figure 1.

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In a further embodiment of the invention said domain consists of the amino acid sequence +1 to about +240 of the sequence presented in Figure 2, or part thereof. Preferably said polypeptide consists of sequence +30 to +60; +88 to +112; or +139 to +175 of the sequence presented in Figure 2.

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In a further preferred embodiment of the invention said domain consists of the amino acid sequence +1 to about +262 of the sequence presented in Figure 3, or part thereof. Preferably said polypeptide consists of sequence +30 to +71; +110 to +140; or +160-+197 of the sequence presented in Figure 3.

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In a further preferred embodiment of the invention said domain consists of the amino acid sequence +1 to about +258 of the sequence presented in Figure 4, or part thereof. Preferably said polypeptide consists of sequence +68 to +90; +105 to +135; or +153 to +191 of the sequence presented in Figure 4.

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In a further embodiment of the invention said polypeptide fragment consists of the amino acid sequence +78 to +105; +128 to +156; or +184 to +227 of the sequence presented in Figure 5, or part thereof.

In a further preferred embodiment of the invention said polypeptide fragment consists of the amino acid sequence +54 to +88; +124 to +150; or +178 to +220 of the sequence presented in Figure 6, or part thereof.

- 5 In a further preferred embodiment of the invention said polypeptide fragment consists of the amino acid sequence +46 to +86; +115 to +142; or +169 to +213 of the sequence presented in Figure 7, or part thereof.

- 10 In a further preferred embodiment of the invention said polypeptide fragment consists of the amino acid sequence +30 to +230; +235 to +270; or +280 to +330 of the sequence presented in Figure 8, or part thereof.

- 15 In a further preferred embodiment of the invention said polypeptide fragment consists of the amino acid sequence +29 to +73; +88 to +124; or +130 to +180 of the sequence presented in Figure 9, or part thereof.

- In a further preferred embodiment of the invention said polypeptide fragment consists of the amino acid sequence +46 to +86; +115 to +142; or +169 to +213 of the sequence presented in Figure 10, or part thereof.

- 20 In a further preferred embodiment of the invention said polypeptide fragment consists of the amino acid sequence +53 to +80; +92 to +123; or +145 to +190 of the sequence presented in Figure 11, or part thereof.

- 25 In a preferred embodiment of the invention said polypeptide is at least 10 amino acids in length.

- In a further preferred embodiment of the invention the length of said polypeptide is at least 20 amino acids; 30 amino acids; 40 amino acids; 50 amino acids; 60 amino acids; 70 amino acids; 80 amino acids; 90 amino acids. Preferably said polypeptide is at least 100 amino acids in length.

In a further preferred embodiment of the invention said polypeptide is a variant polypeptide.

A variant, i.e. a polypeptide or fragment and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions, truncations which may be present in any combination. Among preferred variants are those that vary from a reference polypeptide by conservative amino acid substitutions. Such substitutions are those that substitute a given amino acid by another amino acid of like characteristics. The following non-limiting list of amino acids are considered conservative replacements (similar): a) alanine, serine, and threonine; b) glutamic acid and aspartic acid; c) asparagine and glutamine d) arginine and lysine; e) isoleucine, leucine, methionine and valine and f) phenylalanine, tyrosine and tryptophan. Most highly preferred are variants which retain the same biological function and activity as the reference polypeptide from which it varies.

A functionally equivalent polypeptide according to the invention is a variant wherein one in which one or more amino acid residues are substituted with conserved or non-conserved amino acid residues, or one in which one or more amino acid residues includes a substituent group. Conservative substitutions are the replacements, one for another, among the aliphatic amino acids Ala, Val, Leu and Ile; interchange of the hydroxyl residues Ser and Thr; exchange of the acidic residues Asp and Glu; substitution between amide residues Asn and Gln; exchange of the basic residues Lys and Arg; and replacements among aromatic residues Phe and Tyr.

In addition, the invention features polypeptide sequences having at least 75% identity with the polypeptide sequences as hereindisclosed, or fragments and functionally equivalent polypeptides thereof. In one embodiment, the polypeptides have at least 85% identity, more preferably at least 90% identity, even more preferably at least 95% identity, still more preferably at least 97% identity, and most preferably at least 99% identity with the amino acid sequences illustrated herein.

Furthermore, said polypeptide variants and reference polypeptides are characterised by their binding affinity for a target molecule. For example polypeptides which have binding equilibrium constants of at least about 10^7 M^{-1} , more preferably at least about 10^8 M^{-1} , and most preferably at least about 10^9 M^{-1} .

The polypeptides according to the invention may comprise one or more modified amino acids which preferably improve the anti-bacterial efficacy of said polypeptide. It will be apparent to one skilled in the art that modified amino acids include, by way of example and not by way of limitation, 4-hydroxyproline, 5-hydroxylysine, N⁶-acetyllysine, N⁶-methyllysine, N⁶,N⁶-dimethyllysine, N⁶,N⁶,N⁶-trimethyllysine, cyclohexylalanine, D-amino acids, ornithine. Other modifications include amino acids with a C₂, C₃ or C₄ alkyl R group optionally substituted by 1, 2 or 3 substituents selected from halo (eg F, Br, I), hydroxy or C₁-C₄ alkoxy. Modifications may also include acetylation and/or amidation to amino and/or carboxyl-terminal amino acids to improve *in vivo* stability.

According to an embodiment of the invention there is provided a polypeptide selected from the group consisting of:

- i.) a polypeptide comprising the amino acid sequence of Figure 5, 6, 10 or 11;
 - ii.) a polypeptide comprising an amino acid sequence having at least 75% identity to the polypeptide in (i) and which has anti-bacterial activity;
 - iii.) fragments or variants of the polypeptides in (i) and (ii)
- for use as a therapeutic agent.

In a preferred embodiment the polypeptide comprises the sequence of Figure 5 or Figure 11. In a particularly preferred embodiment the polypeptide comprises the sequence of Figure 5.

In a further embodiment the polypeptide comprises an amino acid sequence having at least 95% identity to the amino acid sequence of Figure 6.

Preferably said polypeptide consists of amino acid sequences as represented by Figures 5, 6, 10 or 11.

It is well within the capability of the skilled artisan to manufacture the polypeptides or peptides according to the invention. These can be made by standard peptide synthesis using a peptide synthesizer or alternatively by recombinant techniques known in the art. For example techniques disclosed in Sambrook et al (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory, Cold Spring Harbour, NY

and references therein; Marston, F (1987) DNA Cloning Techniques: A Practical Approach Vol III IRL Press, Oxford UK; DNA Cloning: and F M Ausubel et al, Current Protocols in Molecular Biology, John Wiley & Sons, Inc.(1994).

- 5 The term polypeptide used in this text means, in general terms, a plurality of amino acids residues joined together by peptide bonds. It is used interchangeably and means the same as protein or peptide.

According to a further aspect of the invention there is provided nucleic acid encoding
10 the polypeptides of the invention for use as a therapeutic agent. The nucleic acid may be isolated or recombinant and may be in substantially pure form.

According to a further aspect of the invention there is provided a therapeutic composition comprising a polypeptide according to the invention.

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In a preferred embodiment of the invention said composition further comprises a carrier.

The polypeptide or polypeptide compositions may be combined, if desired, with a
20 pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier" as used herein means one or more compatible solid or liquid fillers, diluents or encapsulating substances which are suitable for administration into a human. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application.

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The pharmaceutical compositions may contain suitable buffering agents, including: acetic acid in a salt; citric acid in a salt; boric acid in a salt; and phosphoric acid in a salt.

30 The pharmaceutical compositions also may contain, optionally, suitable preservatives, such as: benzalkonium chloride; chlorobutanol; parabens and thimerosal.

The pharmaceutical compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy. All

methods include the step of bringing the active agent into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the active compound into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Compositions suitable for oral administration may be presented as discrete units, such as capsules, tablets, lozenges, each containing a predetermined amount of the active compound. Other compositions include suspensions in aqueous liquids or non-aqueous liquids such as a syrup, elixir or an emulsion.

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous or non-aqueous preparation of polypeptides which is preferably isotonic with the blood of the recipient. This preparation may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation also may be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butane diol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid may be used in the preparation of injectables. Antiseptic mouth wash is an example of a composition according to the invention.

According to a further aspect of the invention there is provided a method to treat a bacterial infection comprising administering to an animal, preferably a human, an amount of a polypeptide according to the invention sufficient to inhibit the progression of disease caused by said bacteria.

In a preferred method of the invention said bacterial infection is a respiratory infection.

In a preferred method of the invention said bacterial infection is caused by a bacterial species selected from the group consisting of: *Staphylococcus aureus*; *Staphylococcus epidermidis*; *Enterococcus faecalis*; *Mycobacterium tuberculis*; *Streptococcus* group B; *Streptococcus pneumoniae*; *Helicobacter pylori*; *Neisseria gonorrhea*;
 5 *Streptococcus* group A; *Borrelia burgdorferi*; *Coccidioides immitis*; *Histoplasma sapsulatum*; *Neisseria meningitidis* type B; *Shigella flexneri*; *Escherichia coli*; *Haemophilus influenzae*; *Mycoplasma pneumonia*; *Pseudomas aeruginosa*; *legionella* sp; *Moraxella catarrhalis*; *Klebsiella pneumoniae*; *Fusobacterium nucleatum*; *Porphyromonas gingivalis*.

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In a further preferred method of the invention said infection is selected from the group consisting of: septicemia; tuberculosis; bacteria-associated food poisoning; blood infections; peritonitis; endocarditis; sepsis; meningitis; pneumonia; stomach ulcers; gonorrhoea; strep throat; streptococcal-associated toxic shock; necrotizing fasciitis;
 15 impetigo; histoplasmosis; Lyme disease; gastro-enteritis; dysentery; shigellosis; periodontal disease.

According to a further aspect of the invention there is provided the use of a polypeptide according to the invention for the manufacture of a medicament for use in
 20 the treatment of a bacterial infection.

In a preferred embodiment of the invention said use is the treatment of infections caused by a bacterial species selected from the group consisting of: *Staphylococcus aureus*; *Staphylococcus epidermidis*; *Enterococcus faecalis*; *Mycobacterium*
 25 *tuberculis*; *Streptococcus* group B; *Streptococcus pneumoniae*; *Helicobacter pylori*; *Neisseria gonorrhea*; *Streptococcus* group A; *Borrelia burgdorferi*; *Coccidioides immitis*; *Histoplasma sapsulatum*; *Neisseria meningitidis* type B; *Shigella flexneri*; *Escherichia coli*; *Haemophilus influenzae*; *Mycoplasma pneumonia*; *Pseudomonas aeruginosa*; *Legionella* sp; *Moraxella catarrhalis*; *Klebsiella pneumoniae*.

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In a further preferred embodiment of the invention said infection is selected from the group consisting of: septicemia; tuberculosis; bacteria-associated food poisoning; blood infections; peritonitis; endocarditis; sepsis; meningitis; pneumonia; stomach ulcers; gonorrhoea; strep throat; streptococcal-associated toxic shock; necrotizing

fasciitis; impetigo; histoplasmosis; Lyme disease; gastro-enteritis; dysentery; shigellosis; periodontal disease.

Preferred features for each aspect of the invention are as for the first aspect indicated
5 *mutatis mutandis*.

The invention will now be further described by way of reference to the following
Figures and Example which are provided for the purposes of illustration only and are
not to be construed as being limiting on the invention. Reference is made to a number
10 of Figures in which:

Figure 1 represents the amino acid sequence of the LPLUNC 1 polypeptide;

Figure 2 represents the amino acid sequence of the LPLUNC 2 polypeptide;
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Figure 3 represents the amino acid sequence of the LPLUNC 3 polypeptide;

Figure 4 represents the non full-length amino acid sequence of the LPLUNC 4
polypeptide;
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Figure 5 represents the amino acid sequence of SPLUNC 1 polypeptide;

Figure 6 represents the amino acid sequence of SPLUNC 2 polypeptide;

25 Figure 7 represents the amino acid sequence of SPLUNC 3 polypeptide;

Figure 8 represents the full length amino acid sequence of the LPLUNC 4
polypeptide;

30 Figure 9 represents the amino acid sequence of the LPLUNC6 polypeptide;

Figure 10 (a) represents the revised (cf. Fig 7) amino acid sequence of the
SPLUNC3(a) polypeptide which arises from the splicing of a 64 basepair exon. The

sequence shown in bold replaces the sequence shown in brackets (below) which corresponds to the sequence in the original SPLUNC3 sequence in Fig 7;

- (b) represents the amino acid sequence of SPLUNC3(b) which is the product of splicing of a 102bp exon 7 that generates an inframe stop codon and removes the second conserved cysteine from the protein.

Figure 11 represents the amino acid sequence of the SPLUNC 7 polypeptide;

- Figure 12 Conservation and diversity in the PLUNC and BPI families. a-e, Sequence alignments created by 3DPSSM coloured according to secondary structure. Helices are shown in yellow, strands in green, and irregular structure in grey. The groups of proteins are (a) N terminal domains of BPI family proteins, (b) C terminal domains of BPI family proteins, (c) C terminal domains of LPLUNC proteins, (d) N terminal domains of LPLUNC proteins and (e) SPLUNC proteins. The sequence in the top line of each group, BPI(X-ray), represents the most confident match of the 3DPSSM program in all cases. The other sequences in each group are the query sequences submitted individually to 3DPSSM. These sequences are shown aligned to BPI with gaps introduced into both sequences of 3DPSSM pairwise alignments as necessary to allow all sequences in a group to be displayed aligned to one representation of the BPI sequence. Where the query sequences align to an insert in the BPI(X-ray) sequence, the relative alignment of the query sequences is simply left justified within the available space. The sequence in the second line of groups a and b is BPI which was also submitted to 3DPSSM for completeness and as a control. The BPI(X-ray) sequence is coloured according to the experimentally determined structure of BPI as evaluated by 3DPSSM, whereas the other sequences are coloured according to the 3DPSSM predicted secondary structure. The start points of the N-terminal domain alignments are offset to facilitate comparison. The full length BPI sequence is numbered from -26, consistent with the numbering used previously(17). f,g, The degree of secondary structure conservation predicted by 3DPSSM in the PLUNC family, mapped onto the structure of BPI. Regions in red are poorly conserved at the secondary structure level (see Methods). (f) Data for the SPLUNC proteins mapped onto the N-domain of BPI, (g) Data for the LPLUNC proteins mapped onto the full length of BPI. Figures f and g were created using Molscrip(31), using the coordinates 1ewf.pdb(18); and

Figure 13 illustrates the expression of the human *PLUNC* genes in nasal, pulmonary and salivary gland tissue.

- a. Total RNA isolated from human nasal septal epithelium, lung and submandibular gland tissue was resolved on denaturing agarose gels, Northern blotted and hybridized with random primed cDNA probes as previously described. Replicate blots were hybridised with ³²P labelled cDNA probes corresponding to *SPLUNC2* and *LPLUNC1-4*. The lower panel shows ethidium bromide staining of a transferred blot. b.

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Figure 14 illustrates the expression of the human *LPLUNC1* gene in human tissues. commercial multiple tissue poly A⁺ dot blot (Clontech, No 7770-1) containing RNA from 50 human tissues, was hybridized with a random primed *LPLUNC1* cDNA probe. The arrows indicate samples producing significant specific hybridization;

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Figure 15 represents the genomic organisation of the human *PLUNC* locus and exon conservation of the *PLUNCs* and related genes. The complete human *PLUNC* gene locus is contained within approximately 300Kb on chromosome 20q 11.2, indicated by the boxed segment of the chromosome. The shaded boxes indicate the relative positions of the seven genes, including *SPLUNC1*. *LPLUNC* genes are indicated by diagonal shading and *SPLUNC* genes by the black stipples. The location of human *RYF3* at the end of the locus is indicated by the white stippled box. The size bar represents 20 Kb;

- 25 Figure 16 illustrates the immunoreactive in vitro translation products detected on a Western blot using the V5 epitope tag antibody. *In vitro* translated hLPLUNC1 and mSPLUNC5 were generated from the flip-in expression clones by the use of the coupled transcription and translation system from Promega. Sample of the resultant products were resolved on SDS-PAGE gels and western blotted using the V5 epitope tag antibody. Immunoreactive products were detected using the ECL detection system from Amersham;

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Figure 17 illustrates *PLUNC* protein expression in stable cell lines expressing hSPLUNC1 (hSP1), empty vector control (Ev), The N-terminal of hLPLUNC1

(hLP1N) and mouse SPLUNC5 (mSP5). Stable lines in which the plasmids were stably integrated were selected by growth in the appropriate antibiotic selection medium. Following a number of passages, cell extracts were prepared and western blotted using the anti-V5 antibody to detect the fusion proteins;

5

Figure 18 illustrates PLUNC protein expression in media from stable cell lines expressing hSPLUNC1 (hSP1), the N-terminal of hLPLUNC1 (hLP1N) and mouse SPLUNC5 (mSP5). Stable lines in which the plasmids were stably integrated were selected by growth in the appropriate antibiotic selection medium. Following a number of passages cell extracts and conditioned medium were prepared and western

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blotted using the anti-V5 antibody To detect the fusion proteins; and

Figure 19 illustrates PLUNC protein purification by metal chelate chromatography of the conditioned medium from stable cell lines expressing hSPLUNC1 (hSP1), N-terminal of hLPLUNC1 (hLP1N) and mSP1unc5. Purified proteins were isolated by

15

metal chelate chromatography and subsequently western blotted using the anti-V5 antibody to detect the fusion proteins

Figure 20 illustrates the organisation of the human PLUNC gene locus. The stippled and black boxes represent "long" (LP) and "short" *PLUNCs* (SP). The position of the human *BASE* gene (*SPLUNC7*) is also shown adjacent to *SPLUNC3*. The size marker represents 50Kb. The PLUNC locus spans a 315kb region of Chromosome 20q11.2 between 31.314 and 31.616Mb.

20

25

Materials and Methods

Characterisation of *PLUNC* genes

PLUNC genes were identified by a combination of nucleotide and protein BLAST searches (blastx and blastn) of the public databases using the human PLUNC sequence (Bingle et al, Biochim Biophys Acta. 1493:363, 2000) as a start point. Individual gene products thus identified were themselves used for further searches. ESTs identified by such searching were assembled into contigs. Where required for

30

completion of sequences individual Image clones were obtained from the MRC HGMP, Cambridge, UK and sequenced using an ABI 377 sequencer with appropriate flanking or specific oligonucleotides as required. The genomic structure of individual genes was determined from genomic sequence from the chromosome 20 sequencing project. Subsequent to our studies the PLUNC gene structures and predicted protein products have appeared in the chromosome 20 sequence. With the exception of the genomic structure of SPLUNC3 our determination is in agreement with the annotation assigned in Genbank.

10 **RNA Isolation and expression studies**

Total RNA was isolated from human nasal septal epithelium, lung and submandibular gland using the RNeasy system (Qiagen). Samples were resolved on replicate denaturing agarose gels, Northern Blotted and hybridized with random primed cDNA probes as previously described. The PLUNC probes corresponded to either ESTs or to RT-PCR fragments. RT-PCR analysis was performed using primer pairs to all of the PLUNCs with 1ug of both nasal septal epithelium and lung RNA as the starting templates for oligo dT priming. Appropriately sized products were directly cloned in TOPO pCRII and sequenced for verification. LPLUNC1 expression was also investigated in a wider range of tissues by hybridizing a multiple tissue RNA dot blot containing RNA from 54 different human tissues (Clontech; Number 7770-1).

Sequence alignment and structural analysis

25 BLAST and PSI-BLAST were performed via the National Centre for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/BLAST>) and used the non redundant sequence database. PSI-BLAST searches used the default threshold of $E < 0.005$ and were iterated to convergence. Sequences that fell outside this tolerance were analysed up to an E value of 10 to check that missing sequences were not marginally detected. Sequences were individually submitted to 3DPSSM (<http://www.bmm.icnet.uk/~3dpssm/>) (Kelly et al, J. Mol. Biol. 299:499, 2000). An in-house python script was used to bring the matched BPI sequences in a group of pairwise alignments into register by inserting gaps in both sequences of individual pairwise alignments as necessary. The classification of residue positions as conserved

or variable was performed as follows. Each position in the alignment of a group of proteins (as in Fig12) was taken in turn. If this position corresponded to a gap in the BPI-match sequence then the position was not classified. Otherwise the predicted secondary structure for the query sequences were compared to the experimental
5 secondary structure of BPI. If there was agreement for 3 or more of the long proteins (2 or more for the short proteins) then the position was denoted conserved. Otherwise the position was denoted variable. Gaps in the query sequence were counted as a secondary structure mismatch. According to the EVA project (<http://cubic.bioc.columbia.edu/eva/sec/common.html>), the PSIPRED method
10 currently has a 3-state accuracy (Q3) of 76%. This is very close to the value observed here for BPI (82%). PSIPRED uses a PSI-BLAST procedure to produce a sequence profile upon which the prediction is made, and thus the predictions for a homologous family are correlated to an extent. However, the PSI-BLAST search is restricted to avoid sequence drift, and thus the profile obtained is quite strongly biased towards the
15 input sequence. Pairwise sequence identities were evaluated using simple sequence directed alignments using clustalw v1.7 in a pairwise fashion.

Accession Numbers for PLUNC proteins

20 The individual PLUNC gene products are represented in the database with the following GI numbers: SPLUNC1 7958616, SPLUNC2 9801234, LPLUNC1 14772576, LPLUNC2 11877274, LPLUNC3 11877275, LPLUNC4 11877276, LPLUNC4 (Full length) AX283507, LPLUNC6 XM-066207, and SPLUNC7 NM_173859. The deduced sequence of SPLUNC3 was determined from multiple
25 ESTs represented by BG717907 or AW340528

Generation of PLUNC protein expression constructs

For generation of PLUNC proteins the FLP-In expression system from Invitrogen was
30 used. The FLP-In System allows integration and expression of genes in mammalian cells at a specific genomic location. The FLP-In System involves introduction of a FLP Recombination Target (FRT) site into the genome of the mammalian cell line of choice. The expression vector containing the PLUNC gene is then integrated into the genome via FLP recombinase-mediated DNA recombination at the FRT site.

PLUNC protein expression constructs were generated by PCR using standard techniques. The forward primers were designed to contain a functional Kozak site around the initiating ATG. The reverse primers were designed to miss out the stop
5 codon and allow continuation of translation into the epitope tags of the expression vector.

pcDNA5/FRT/V5-His-TOPO was used as the expression vector. pcDNA5/FRT/V5-His-TOPO is a 5.1 kb expression vector designed to facilitate rapid cloning and
10 expression of PCR products using the Flp-In system. When cotransfected with the pOG44 Flp recombinase expression plasmid into a Flp-In mammalian host cell line, the pcDNA5/FRT/V5-His-TOPO vector containing the PCR product of interest is integrated in a Flp recombinase-dependent manner into the genome. The pcDNA5/FRT/V5-His-TOPO vector contains the following elements: The human
15 cytomegalovirus (CMV) immediate-early enhancer/promoter for high-level constitutive expression of the gene of interest in a wide range of mammalian cells. A TOPO Cloning site for rapid and efficient cloning of *Taq*-amplified PCR products. A C-terminal peptide containing the V5 epitope and a polyhistidine (6xHis) tag for detection and purification of recombinant protein. A FLP Recombination Target
20 (FRT) site for Flp recombinase-mediated integration of the vector into the Flp-In host cell line. A Hygromycin resistance gene for selection of stable cell lines.

Following the PCR reaction, products were checked by standard agarose gel electrophoresis. The products were then cloned into the expression vector in the
25 following manner. Fresh PLUNC PCR product (0.5 μ l), Salt Solution (1 μ l) and Sterile Water (to a final volume of 5 μ l) were mixed with TOPO vector (1 μ l) and incubated for 5 minutes at room temperature (22-23°C). The reaction was then placed on ice prior to chemical transformation. For transformation 2 μ l of the TOPO Cloning reaction was added into a vial of One Shot® TOP10 Chemically Competent *E. coli*
30 and mixed. The reaction was then incubated on ice for 5 to 30 minutes. The cells were Heat-shockd for 30 seconds at 42°C without shaking and transferred to ice. 250 μ l of room temperature SOC medium was added and the tube shaken at 37°C for 1 hour. 25 μ l from each transformation was spread on a prewarmed selective plate and incubate overnight at 37°C. Positive colonies were picked for miniprep analysis and

sequencing.

In vitro Translation of PLUNC proteins

- 5 Following identification of the correct construct sequences, coupled transcription and translation assays were then used to generate recombinant protein for further analysis. The TNT® Quick Coupled Transcription/Translation System (from Promega) is a convenient single-tube, coupled transcription/translation reactions for eukaryotic *in vitro* translation.
- 10 The reaction was performed as follows: Mix together TNT® Quick Master Mix (40µl); Methionine, 1mM (1µl) plasmid DNA template, 0.5µg/µl (2µl) and Nuclease-Free Water to a final volume of 50µl. Incubate the reaction at 30°C for 60–90 minutes and analyze the results by SDS-PAGE using standard protocols.
- For Western blot analysis of the translated proteins, the proteins were transferred from
15 the gel onto nitrocellulose or PVDF membrane by electrophoretic transfer. The blot was then be subjected to immunodetection analysis.

Immunoblotting of PLUNC proteins

- 20 The PLUNC expression constructs have been designed with a V5 epitope tag on the C-terminus which can be used to detect the fusion proteins in the Western Blot analysis using the following standard protocols:
- Incubate the blot in 10 ml blocking buffer using a rocker platform for 1 hour at room temperature. Wash the nitrocellulose membrane in 20 ml PBST 2X for 5 minutes each
25 with gentle agitation. Transfer membrane to a tray containing the Anti-V5 Antibody diluted 1:5000 in 10 ml blocking buffer and incubate at room temperature with gentle agitation for 1-2 hours. Transfer membrane to a tray containing 20 ml PBST and wash for 2 x5 minutes with gentle agitation. Transfer membrane to a clean tray and add secondary antibody (HRP-conjugated) diluted 1:1000 into blocking buffer. Incubate
30 with gentle agitation for 1 hour. Wash for 2 x 5 minutes and detect the proteins using the ECL system (Amersham).

Establishment of cell lines expressing PLUNC proteins

The Flp-In System allows integration and expression of the PLUNC genes in mammalian cells at a specific genomic location. Flp-In CHO cells stably express a single integrated Flp Recombination Target (FRT) site and are the start point for generation of stable cell lines by Flp mediated recombination.

- 5 Generation of Flp-In expression cell lines requires cotransfection of the Flp-In cell line with a Flp-In expression vector containing a PLUNC gene and the Flp recombinase expression plasmid, pOG44. Flp recombinase mediates insertion of Flp-In PLUNC expression construct into the genome at the integrated FRT site through site-specific DNA recombination. Stable cell lines expressing the Flp-In PLUNC
10 expression vector were be generated by selection using hygromycin B.

Flp-In-CHO were grown in Ham's F12, 10% FBS, 2 mM L-glutamine, 1% Pen-Strep and 100 µg/ml Zeocin using standard protocols.

- Transient transfection was performed using Lipofectamine (Gibco) with a 9:1 ratio of pOG44:pcDNA5/FRT plasmid DNA. 24 hours after transfection, the cells were
15 washed and fresh medium added. 48 hours after transfection, the cells were split into fresh medium. The cells were incubated at 37°C for 2-3 hours until they attached to the culture dish.

- The medium was removed and fresh medium containing hygromycin added. The cells were refed selective medium every 3-4 days until foci were identified. The
20 hygromycin-resistant foci were expand and the resultant cells were tested for expression of the PLUNC protein in the cell pellet by western blotting.

Identification of PLUNC proteins in conditioned medium

- 25 After confirmation of the expression of the PLUNC proteins in the stable cells, the proteins were isolated from the conditioned medium taking advantage of the fact that the proteins have a His tag the extreme C-terminal end of the proteins. This tag can be used as an affinity tag fro purification with metal chelate resins. The ProBond Purification System (Invitrogen) was used to purify 6xHis-tagged recombinant
30 proteins expressed in bacteria, insect and mammalian cells. The system is designed around the high affinity and selectivity of ProBond resin for recombinant fusion proteins that have been tagged with six tandem histidine residues.

Proteins were isolated under native conditions: For this procedure 1ml of resin is added to 50 ml of conditioned medium. The solution is mixed for 30-60 minutes

using gentle agitation to keep the resin suspended. The resin is settled and washed in Native Wash Buffer at least three times. Finally the resin is settled into a column and the protein eluted with Native Elution Buffer and analyzed by SDS-PAGE and Western blotting.

5

Direct binding of PLUNC proteins to microbes

The direct interaction of PLUNC proteins with whole bacteria is detected using standard published methods (Jack et al., Journal of Infectious Diseases; 184:1152-1162, 2001).

10

PLUNC proteins (anti V5 and His tagged - either purified or in conditioned medium) are directly interacted with a variety of bacteria. Organisms are incubated at 37°C for 15 min and then centrifuged at 2500 g. The cell pellet is resuspended in anti V5 antibody. After a 20-min incubation at 37°C, the samples are centrifuged and washed and then resuspended in a secondary antibody labelled with either phycoerythrin (PE) or fluorescein isothiocyanate (FITC). The samples are incubated for a further 20 min at 37°C, centrifuged, washed, and fixed in 1% vol/vol formaldehyde and 1% wt/vol glucose for flow cytometry.

15
20

Anti-inflammatory function of PLUNC proteins

The anti inflammatory function of PLUNC proteins is tested for by the ability of purified proteins or conditioned medium to block the synthesis of TNF α and IL-8 by human macrophages (Pridmore et al., Journal of Infectious Diseases, 183:89-96, 2001).

25

Peripheral blood mononuclear cells are obtained from healthy volunteers and prepared by density gradient centrifugation. Cells are seeded at 10⁶/well in 24-well culture plates in 1 mL of RPMI 1640 supplemented with 2 mM L-glutamine and 10% fetal calf serum at 37°C in 5% CO₂ in humidified air. After 24 h, the cells are washed to remove the nonadherent population, and then reincubated with 2 mL of serum-containing medium. Bacterial inocula are pelleted, washed, declumped by vortexing in the presence of 3-mm glass beads (Fisher Scientific), and resuspended in

30

Dulbecco's modified Eagle medium before being added to MDM; bacterial counts are adjusted spectrophotometrically. Viable counts are checked by a serial dilution technique. Bacterial suspensions of various concentrations in 50 L of DMEM are added, and cells incubated at 37°C in 5% CO₂ in humidified air for 2 h. After
5 incubation, samples of media are removed from cells and immediately cooled to -70°C. After removing cellular material in cell supernatants by centrifugation, TNF α and IL-8 is measured by ELISA.

Interference by PLUNC proteins of LPS/LBP interactions.

10

The ability of purified PLUNC proteins or conditioned medium to inhibit binding of endotoxin to LBP ie, the most important step in the biological response to endotoxin, is tested. The assay (Endblock from HBT, based on the Scott et al., J. Immunol. 164: 549-553, 2000) is used to detect anti-LPS peptides and proteins that inhibit the
15 biological activity of LPS. The assay is based on an antibody to LBP that is reactive with LBP of a wide variety of animals. This antibody interacts with LBP in such a way that LBP is still highly reactive with endotoxin. The assay is based upon the inhibition principle: addition of a compound that competes with LBP for binding to LPS leads to reduction of binding of labeled LPS which is detected by a HRP labelled
20 conjugate.

Anti bacterial effects of PLUNC proteins

A variety of assays such as described in Cole et al., The Journal of Immunology, 169: 6985-6991, 2002 are used to detect antibacterial effects of purified PLUNC proteins
25 of conditioned medium;

Radial diffusion assay

30 In this assay the underlay consists of 1% agarose and 1/100 dilution of TSB in 10 mM sodium phosphate (pH 7.4), either alone or supplemented with 50 or 100 mM NaCl. The overlay consists of 6% TSB and 1% agarose in dH₂O for all assays. Bacteria (4×10^6) are mixed with 10 ml of underlay gel solutions kept molten at 48°C and

poured into 100-cm² square petri dishes. A series of 3.2-mm diameter wells are punched after the agarose solidified, and 5 μ l of PLUNC protein samples are added into designated wells. Plates are incubated at 37°C for 3 h to allow for peptide diffusion. The microbe-laden underlay is then covered with 10 ml of molten overlay, and the plates are allowed to harden. Antibacterial activity is identified as a clear zone around the well absent of microbial growth after 18-h incubation at 37°C.

Gel-overlay assay

The gel-overlay assay measures the activity of peptides and proteins that diffuse from an electrophoresed PAGE gel into an agarose gel embedded with microcolonies of bacteria. PLUNC proteins are separated by AU-PAGE. The gels are washed 20 min in 10 mM sodium phosphate pH 7.4 then placed on a premade 1% agarose plate containing 10 mM sodium phosphate with 100 mM NaCl (pH 7.4), 0.03% (w/v) of TSB powder, and 4×10^6 bacteria. The plate is then incubated at 37°C for 3 h to allow the proteins and peptides in the polyacrylamide gel to diffuse into the underlying bacterial layer. The polyacrylamide gel is then removed, and the bacterial layer is overlaid with a nutrient layer that contained 6% TSB in 1% agarose. Clear zones without bacterial growth represent antibacterial activity.

Colony forming unit (CFU) microassays

Bacteria (OD₆₂₅ = 0.2) are diluted 100- to 300-fold in HBSS for use in the CFU microassay. Samples consist of 6 μ l of bacterial dilution in HBSS/0.2%TSB plus 24 μ l of conditioned medium or purified PLUNC proteins. Separate tubes with 6 μ l of bacteria and 24 μ l of control medium are used as controls for microbial growth. Two microliters of the sample (or buffer-only control) are loaded into each of 12 wells by pipetting directly underneath liquid wax (to avoid evaporation). The plate is incubated at 37°C/5% CO₂. At specific time points, samples from the wells are plated on tryptic soy agar plates, and CFUs are counted following overnight incubation at 37°C.

The invention will now be illustrated by the following non limiting example;

Example

Stable cell lines which express human SPLUNC1, hLPLUNC1 and the N-terminal of hLPLUNC1 were prepared.

5

The ability of each clone to generate the appropriate product was tested by *in vitro* translation followed by western blotting using an antibody to the V5 epitope tag at the C-terminal end of the protein. Such analysis confirmed that the protein must be appropriately synthesised *in vitro* or the epitope tag would not be generated and
10 detected (see Fig 16).

Successful establishment of lines was initially followed by western blotting of cell extracts with the V5 antibody (Fig 17)

15 Following establishment of these lines it was shown that these proteins are secreted from the cells. Soluble secreted proteins were collected for functional assays. Conditioned medium was taken from the cells and concentrated using molecular cut off membranes and the products detected in the medium by western blotting (Fig 18).

20 Purified recombinant PLUNC proteins were also purified by the use of metal chelate chromatography with binding of the His tag of the fusion protein to the affinity support (Fig 19).

CLAIMS

1. A polypeptide selected from the group consisting of:
 - iv.) a polypeptide comprising the amino acid sequence of Figure 5, 6, 10 or 11;
 - 5 v.) a polypeptide comprising an amino acid sequence having at least 75% identity to the polypeptide in (i) and which has anti-bacterial activity;
 - vi.) fragments or variants of the polypeptides in (i) and (ii)for use as a therapeutic agent.
- 10 2. A polypeptide as claimed in claim 1 wherein the polypeptide comprises the amino acid sequence of Figure 5.
3. A polypeptide as claimed in claim 1 wherein the polypeptide comprises an amino acid sequence having at least 95% identity to the amino acid sequence of
15 Figure 6.
4. A polypeptide as claimed in claim 1 wherein the polypeptide is the amino acid sequence of Figure 5, 6, 10 or 11.
- 20 5. A polypeptide as claimed in claim 1 wherein the polypeptide consists of a fragment of the amino acid sequence of Figure 5, 6, 10 or 11.
6. A polypeptide as claimed in claim 5 wherein the polypeptide fragment consists of the amino-terminal domain of the amino acid sequence of Figure 5, 6, 10 or 11.
25
7. A polypeptide as claimed in claim 6 wherein the polypeptide fragment consists of the amino acid sequence +78 to +105; +128 to +156; or +184 to +227 of the sequence presented in Figure 5, or part thereof.
- 30 8. A polypeptide as claimed in claim 6 wherein the polypeptide fragment consists of the amino acid sequence +54 to +88; +124 to +150; or +178 to +220 of the sequence presented in Figure 6, or part thereof.

9. A polypeptide as claimed in claim 6 wherein the polypeptide fragment consists of the amino acid sequence +46 to +86; +115 to +142; or +169 to +213 of the sequence presented in Figure 10, or part thereof.
- 5 10. A polypeptide as claimed in claim 6 wherein the polypeptide fragment consists of the amino acid sequence +53 to +80; +92 to +123; or +145 to +190 of the sequence presented in Figure 11, or part thereof.
11. A therapeutic composition comprising a polypeptide as claimed in any of
10 claims 1 to 10.
12. A composition as claimed in claim 11 further comprising a pharmaceutically-acceptable carrier.
- 15 13. A method to treat a bacterial infection comprising administering to an animal, preferably a human, an amount of a polypeptide as claimed in any of claims 1 to 10 sufficient to inhibit the progression of disease caused by said bacteria.
14. A method as claimed in claim 13 wherein said bacterial infection is a
20 respiratory infection.
15. A method as claimed in claim 14 wherein said bacterial infection is caused by a bacterial species selected from the group consisting of: *Staphylococcus aureus*; *Staphylococcus epidermidis*; *Enterococcus faecalis*; *Mycobacterium tuberculosis*;
25 *Streptococcus group B*; *Streptococcus pneumoniae*; *Helicobacter pylori*; *Neisseria gonorrhea*; *Streptococcus group A*; *Borrelia burgdorferi*; *Coccidioides immitis*; *Histoplasma sapsulatum*; *Neisseria meningitidis type B*; *Shigella flexneri*; *Escherichia coli*; *Haemophilus influenzae*; *Mycoplasma pneumonia*; *Pseudomas aeruginosa*; *legionella sp*; *Moraxella catarrhalis*; *Klebsiella pneumoniae*;
30 *Fusobacterium nucleatum*; *Porphyromonas gingivalis*.
16. A method as claimed in claim 13 wherein the bacterial infection is selected from the group consisting of: septicemia; tuberculosis; bacteria-associated food poisoning; blood infections; peritonitis; endocarditis; sepsis; meningitis; pneumonia;

stomach ulcers; gonorrhoea; strep throat; streptococcal-associated toxic shock; necrotizing fasciitis; impetigo; histoplasmosis; Lyme disease; gastro-enteritis; dysentery; shigellosis; periodontal disease.

- 5 17. The use of a polypeptide as claimed in any of claims 1 to 10 in the manufacture of a medicament for use in the treatment of a bacterial infection.

18. The use as claimed in claim 17 in the treatment of bacterial infections caused by a bacterial species selected from the group consisting of: *Staphylococcus aureus*;
10 *Staphylococcus epidermidis*; *Enterococcus faecalis*; *Mycobacterium tuberculosis*; *Streptococcus group B*; *Streptococcus pneumoniae*; *Helicobacter pylori*; *Neisseria gonorrhea*; *Streptococcus group A*; *Borrelia burgdorferi*; *Coccidioides immitis*; *Histoplasma sapsulatum*; *Neisseria meningitidis type B*; *Shigella flexneri*; *Escherichia coli*; *Haemophilus influenzae*; *Mycoplasma pneumonia*; *Pseudomonas*
15 *aeruginosa*; *Legionella sp*; *Moraxella catarrhalis*; *Klebsiella pneumoniae*.

19. The use as claimed in claim 17 wherein the bacterial infection is selected from the group consisting of: septicaemia; tuberculosis; bacteria-associated food poisoning; blood infections; peritonitis; endocarditis; sepsis; meningitis; pneumonia; stomach
20 ulcers; gonorrhoea; strep throat; streptococcal-associated toxic shock; necrotizing fasciitis; impetigo; histoplasmosis; Lyme disease; gastro-enteritis; dysentery; shigellosis; periodontal disease.

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MAGPWTFTLL CGLLAATLIQ ATLSPTAVLI LGPKVIEKEL TQELKDHNAT
SILQQPLLS AMREKPAGGI PVLGSLVNTV LKHVIWLKVI TANILQLQVK
PSANDQELLV KIPLDMVAGF NTPLVKTIVE FHMTTEAQAT IRMDTSASGP
TRLVLSDCAT SHGSLRIQLL HKLSFLVNAL AKQVMNLLVP SLPNLVKNQL
CPVIEASFNG WYADLLQLVK VPISLSIDRL EFDLLYPAIK GDTIQLYLGA
KLLDSQGKVT KWFNNSAASL TMPTLDNIPF SLIVSQDVVK AAVAAVLSPE
EFMVLLDSVL PESAHRLKSS IGLINEKAAD KLGSTQIVKI LTQDTPEFFI
DQGHAKVAQL IVLEVFPSS ALRPLFTLGI EASSEAQFYT KGDQLILNLN
NISSDRIQLM NSGIGWFQPD VLKNIITEII HSILLPNQNG KLRSGVPVSL
VKALGFEEAE SSLTKDALVL TPASLWKPSS PVSQ

Fig. 1

MAWASRLGLL LALLLPVGA STPGTVVRLN KAALSYVSEI GKAPLQRALQ
VTVPHFLDWS GEALQPTRIR ILNVHVPRLH LKFIAGFGVR LLAAANFTFK
VFRAPELEL TLPVELLADT RVTQSSIRTP VVSISACSLF SGHANEFDGS
NSTSHALLVL VQKHIKAVLS NKLCLISISNL VQGVNVHLGT LIGLNPVGPE
SQIRYSMVSV PTVTSDYISL EVNAVLFLLG KPIILPTDAT PFVLPRHVG
EGSMATVGLS QQLFDSALLL LQKAGALNLD ITGQLRSDDN LLNTSALGRL
IPEVARQFPE PMPVVLKVRL GATPVAMLHT NNATLRLQPF VEVLATASNS
AFQSLFSLDV VVNLRLQLSV SKVKLQGTTT VLGDVQLTVA SSNVGFIDTD
QVRTLMGTVF EKPLLDHLNA LLAMGIALPG VVNLHYVAPE IFVYEGYVVI
SSGLFYQS

Fig. 2

MLALWSLLLL WGLATPCQEL LETVGTLARI DKDELGKAIQ NSLVGEPILQ
NVLGSVTAVN RGLLGSGGLL GGGGLLGHGG VFGVVEELSG LKIEELTLPK
VLLKLLPGFG VQLSLHTKVG MHCSGPLGGL LQLAAEVNVT SRVALAVSSR
GTPILILKRC STLLGHISLF SGLLPTPLFG VVEQMLFKVL PGLLCPVVDS
VLGVNELLG AVLGLVSLGA LGSVEFSLAT LPLISNQYIE LDINPIVKSV
AGDIIDFPKS RAPAKVPPKK DHTSQVMVPL YLFNTTFGLL QTNGALDMDI
TPELVPSDVP LTTTDLAALL PEALGKLPLH QQLLLFLRVR EAPTVTLHNK
KALVSLPANI HVLFYVPGKT PESLFELNSV MTVRAQLAPS ATKLHISLSL
ERLSVKVASS FTHAFDGSRL EEWLSHVGA VYAPKLNVAL DVGIPLPKVL
NINFSNSVLE IVE

Fig. 3

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RLHRRELQPG EIPPGVATGA VGPGGLLGTG GMLAADGILA GQGGLLG GGG
LLGDGGLLGG GGVLGVLGEG GILSTVQGIT GLRIVELTLP RVSVRLLPGV
GVYLSLYTRV AINGKSLIGF LDIAVEVNIT AKVRLTMDRT GYPRLVIERC
DTLLGGIKVK LLRGLLPNLV DNLVNRVLAD VLPDLLCPIV DVVLGLVNDQ
LGLVDSLIP L GILGSVQYTF SSLPLVTGEF LEIDLNTLVG EAGGGLIDYP
LGWPAVSPKP MPELPPMGDN TKSQ LAMSAN FLGSVLTLLQ KQH ALDLDIT
NGMFEELPPL TTATLGALIP KVFQQYPESC PLIIRIQVLN PPSVMLQKDK
ALVKVLATAE VMVSQPKDLE TTICLIDVDT EFLASFSTEG DKLMIDAKLE
KTSLNLR TSN VGNFDIGLME VLVEKIFDLA FMPAMNAV LG SGVPLPKILN
IDFSNADIDV LEDLLVLSA

Fig. 4

MFQTGGLIVF YGLLAQTMAQ FGGLPVPLDQ TLPLNVNPAL PLSPTGLAGS
LTNALSNGLL SGGLLGILEN LPLLDILKPG GGTSGGGLLGG LLGKVT SVIP
GLNNIIDIKV TDPQLLELGL VQSPDGHRLY VTIPLGIKLQ VNTPLVGASL
LRLAVKLDIT AEILAVRDKQ ERIHLVLGDC THSPGSLQIS LLDGLGPLPI
QGLLDSLTGI LNKVLP ELVK GNVCP LVNEV LRGLDITLVH DIVNMLIHGL
QFVIKV

Fig. 5

MLQLWKLVLL CGVLTGTSES LLDNLGNDLS NVVDKLEPVL HEGLETVDNT
LKGILEKLVK DLGVLQKSSA WQLAKQKAQE AEKLLNNVIS KLLPTNTDIF
GLKISNSLIL DVKAEPIDDG KGLNLSFPVT ANVTVAGPII GQIINLKASL
DLLTAVTIET DPQTHQPVAV LGECASDPTS ISLSLLDKHS QIINKFVNSV
INTLKSTVSS LLQKEICPLI RIFIHSLDVN VIQQVVDNPQ HKTQLQTLI

Fig. 6

MMCPLWRLLI FLGALLALPL APHKQPWPGL AQAH RDNKST LARIIAQGLI
KHNAESRIQN IHFGDRLNAS AQVAPGLVGW LISGRKHQQQ QESSINITNI
QLDCGGIQIS FHKEWFSANI SLEFDLELRP SFDNNIVKMC AHMSIVVEFW
LEKDEFGRRD LVIGKCDAEP SSVHVAILTE AIPPKMNQFL YKLKENLQKV
LPHMVESQVC PLIGEILGQL DVKLLKSLIE QEVL MNQPNM KPATSECRLE
SPQ

Fig. 7

3/11

MWMAWCVAALS VVAVCGTSHETNTVLRVTKDVL SNAISGMLQQSDALHSA
LREVPLGVGDIPYND FHV RGPPP VYTNGKKLDGIYQY GHIETNDNTAQLG GK
YRYGEILESEGSIRD LRNSGYRSAENAYGGHRGLGRYRAAPVGR LHRRELQP
GEIPPGVATGAVGPGG LLGTGGMLAADGILAGQGGLLGGGGLLGDGGLLGG
GGVLGVLGEGGILSTVQGITGLRIVELTLP RVSVRLLPGVGVYLSLYTRVAING
KSLIGFLDIAVEVNITAKVRLTMDRTGYPRLVIERC
DTLLGGIKVKLLRG LLPNLVDNLVNRVLADVLPDLLCPIVDVVLGLVNDQLG
LVDSLIP LGILGSVQYTFSSLP LVTGEFLELDLNTLVGEAGGGLIDYPLGWPAV
SPKPMPELPPMGDNTKSQLAM SANFLG SVLTLLQKQHALDLDITNGMFEELP
PLTTATLGALIPKVFQQYP ESCPLIIRIQVLNPPSVMLQKDKALVKVLATAEVM
VSQPKDLETTICLIDVDTEFLASFSTEGDKLMIDAKLEKTSNLNRTSNVGNFDI
GLMEVLVEKIFDLAFMPAMNAV LGSGVPLPKILNIDFSNADIDVLEDLLVLSA

Fig. 8

MLRILCLALCSLLTGTRADPGALLRLGMDIMNQVQSAMDESHILEKMAAEAG
KKQPGMKPIKGITNLKV KDVQLPVITLNFVPGVGIFQCVSTGMTVTGKSFMG
GNMEIIVALNITATNRLLRDEETGLPVFKSEGCEVILVNVKTNLPSNMLPKMV
NKFLDSTLHKVLPGLMCPAIDAVLVYVNRKWTNLSDPMPVGQMGTVKYVL
MSAPATTASYIQLDFSPVWQQQKGKTIKLADAGEALT FPEGYAKGSSQLLLPA
TFLSAELALLQKSFHVNIQDTMIGELPPQT TKT LARFIPEVAVAYPKSKPLTTQI
KIKKPPKVTMKTGKSLLHLHSTLEMFAARWRSKAPMSLFLLEVHFNLKVQYS
VHENQLQMATS LDRLLSLSRKSSSIGNFNERELTG FITSYLEEAYIPV VNDVLQ
VGLPLPDFLAMNYNLAELDIVENALMLDLKL

Fig. 9

MMCPLWRL LIFLGLLALPLAPHKQPWPGLAQAH RDNKSTLARIIAQGLIKHN
AESRIQNIHF GDRLNASAQVAPGLVGWLISGRKHQQQQESSINITNIQLDCGGI
QISFHKEWFSANISLEFDLELRPSFDNNIVKMCAHMSIVVEFWLEKDEFGRRD
LVIGKCDAEPSSVHVAILTEAIPPKMNQFLYNLKENLQKVLPHMVESQVCPLI
GEILGQLDVKLLKSLIEQEAAHEPTHHETSQPSACQAGESPS

Fig. 10(a)

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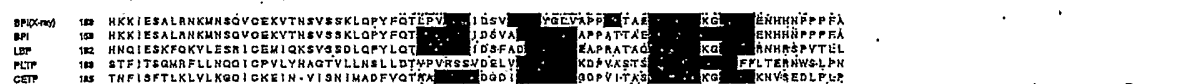
MMCPLWRLLIF LGLLALPLAPHKQPWPGLAQAHARDNKSTLARIIAQGLIKH
NAESRIQNIHFGDRLNASAQ VAPGLVGWLI SGRKHQQQQE SSINITNIQL
DCGGIQISFH KEWFSANISLEFDLELRPSF DNIIKMCAH MSIVVEFWLE
KDEFGRRDLV IGKCD AEPSS VHVAILTEAIPPKMNQFLYN LKENLQKVLP
HMOVESQPLA

Fig. 10(b)

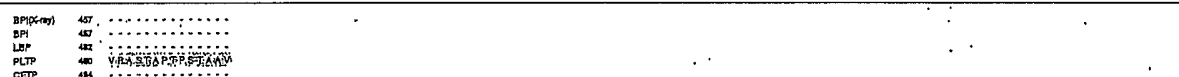
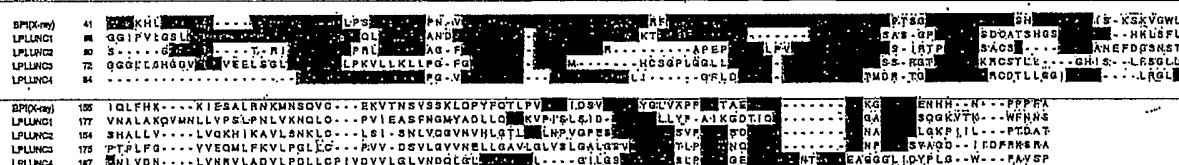
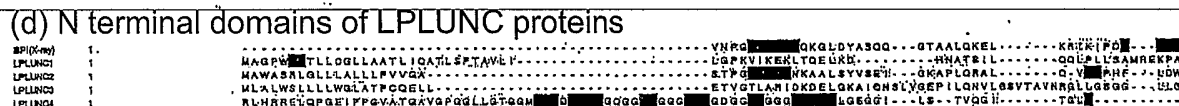
MLNVSGLFVLLCGLLVSSSAQEVLAVGVSSQLLNDLTQGLLRADFLPSLQTTGL
QKPLSSAFDGVSGLLDIFGPPLTNEINTVSIQVKNPQLLHVSIESTPQRKEATQ
VPFTSELIVQLLTMKPFTANMQSDIKVQIRLEKNVGGRYELAFGNCRLPEAI
WIQTGVQLAPAQKFVVANIERNLKNIVAHDLGRKVCPVINKWLYNLDQHVV
KELINLVLLQEKYQVTI

Fig. 11

(a) N terminal domains of BPI family



SP1 (core)	241	FFVMEPPAAADHRL	SDYFNTAGLVYQGAAYL	ADDMFKPKSKFRLTKFFS---	TLPFAVAKKPFMMK	BTFR	BT	A	LPNSA
SP2	242 <th>QVQVQVQVQVQVQV</th> <th>QVQVQVQVQVQVQV</th> <th>QVQVQVQVQVQVQV</th> <th>QVQVQVQVQVQVQV</th> <th>BTFR</th> <th>BT</th> <th>A</th> <th>LPNSA</th>	QVQVQVQVQVQVQV	QVQVQVQVQVQVQV	QVQVQVQVQVQVQV	QVQVQVQVQVQVQV	BTFR	BT	A	LPNSA
SP3	243 <th>AAVSLSPGHHH</th> <th>SDYVNTASLVYHEEQY</th> <th>TDQDIIPDSNLI</th> <th>TKTSF-</th> <th>PPFPLALALYTHNN</th> <th>BTFR</th> <th>BT</th> <th>LPNSA</th>	AAVSLSPGHHH	SDYVNTASLVYHEEQY	TDQDIIPDSNLI	TKTSF-	PPFPLALALYTHNN	BTFR	BT	LPNSA
PLTP	251 <th>AAVPEQIQDEER</th> <th>SEFFQSPAGSYFRAQL</th> <th>VGDNVPHDLDMLLRATY</th> <th>FG--</th> <th>SI-VLLSPAVIDSF</th> <th>BTFR</th> <th>BT</th> <th>LPNSA</th>	AAVPEQIQDEER	SEFFQSPAGSYFRAQL	VGDNVPHDLDMLLRATY	FG--	SI-VLLSPAVIDSF	BTFR	BT	LPNSA
CETP	258 <th>TFSPITLG-DSH</th> <th>SERVFHSKLAKYAFQDRL</th> <th>WQDFEQ---</th> <th>AWLETGFTNHQEI</th> <th>QFQEVVGQFFS-Q</th> <th>BTFR</th> <th>BT</th> <th>LPNSA</th>	TFSPITLG-DSH	SERVFHSKLAKYAFQDRL	WQDFEQ---	AWLETGFTNHQEI	QFQEVVGQFFS-Q	BTFR	BT	LPNSA
SP1 (core)	358 <th>-----</th> <th>GN</th> <th>-----</th> <th>NTGFFPVFELIQIMNYIV</th> <th>ILVLPVFNKELQKQ</th> <th>LF</th> <th>TPAR</th> <th>-----</th>	-----	GN	-----	NTGFFPVFELIQIMNYIV	ILVLPVFNKELQKQ	LF	TPAR	-----
SP2	359 <th>-----</th> <th>GN</th> <th>-----</th> <th>LKLDRLLELKH-SNIGPFFVLELQIMNYIV</th> <th>ILVLPVFNKELQKQ</th> <th>LF</th> <th>TPAR</th> <th>-----</th>	-----	GN	-----	LKLDRLLELKH-SNIGPFFVLELQIMNYIV	ILVLPVFNKELQKQ	LF	TPAR	-----
SP3	360 <th>-----</th> <th>GN</th> <th>-----</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th>	-----	GN	-----	LVVFNKELQKQ	LVVFNKELQKQ	LVVFNKELQKQ	LVVFNKELQKQ	LVVFNKELQKQ
PLTP	361 <th>-----</th> <th>GN</th> <th>-----</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th>	-----	GN	-----	LVVFNKELQKQ	LVVFNKELQKQ	LVVFNKELQKQ	LVVFNKELQKQ	LVVFNKELQKQ
CETP	362 <th>-----</th> <th>GN</th> <th>-----</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th>	-----	GN	-----	LVVFNKELQKQ	LVVFNKELQKQ	LVVFNKELQKQ	LVVFNKELQKQ	LVVFNKELQKQ
SP1 (core)	363 <th>-----</th> <th>GN</th> <th>-----</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th>	-----	GN	-----	LVVFNKELQKQ	LVVFNKELQKQ	LVVFNKELQKQ	LVVFNKELQKQ	LVVFNKELQKQ
SP2	364 <th>-----</th> <th>GN</th> <th>-----</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th>	-----	GN	-----	LVVFNKELQKQ	LVVFNKELQKQ	LVVFNKELQKQ	LVVFNKELQKQ	LVVFNKELQKQ
SP3	365 <th>-----</th> <th>GN</th> <th>-----</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th>	-----	GN	-----	LVVFNKELQKQ	LVVFNKELQKQ	LVVFNKELQKQ	LVVFNKELQKQ	LVVFNKELQKQ
PLTP	366 <th>-----</th> <th>GN</th> <th>-----</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th>	-----	GN	-----	LVVFNKELQKQ	LVVFNKELQKQ	LVVFNKELQKQ	LVVFNKELQKQ	LVVFNKELQKQ
CETP	367 <th>-----</th> <th>GN</th> <th>-----</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th> <th>LVVFNKELQKQ</th>	-----	GN	-----	LVVFNKELQKQ	LVVFNKELQKQ	LVVFNKELQKQ	LVVFNKELQKQ	LVVFNKELQKQ

[illegible]

SPINCLIN1	1	MDPTGGLIVFYGALLAQDMAOFGQGLPYPLDQTEPLNNPFLPSTGGLAGELTNALSGLLGGGLDLEHLENF	VNPG	QKG	LDYASGGGTAAALG	ELKHKIR	D	---
SPINCLIN1	1	MDPTGGLIVFYGALLAQDMAOFGQGLPYPLDQTEPLNNPFLPSTGGLAGELTNALSGLLGGGLDLEHLENF	AC	QSGGGLGGLG	---	VTSP	IV	---
SPINCLIN2	1	MDGLWKVLVGLGATGTSSESLDNLNDLSNVVDKLEPVLNREET	VDHTLKG	ILEKLKVGLGVKLSXWQ	---	LAQKQ	AD	EAEKLL
SPINCLIN2	1	MDGLPWLRLIFLGALLALPLAFHGWPSQAQAH	QKSTLARI	IARG	L	IKHAEASHQNI	HEGG	NARAQVAP
SPINCLIN1	40	GLNNI	SPD	GH	PTAG	GH	---	DSKGVG
SPINCLIN1	101	GLNNI	SPD	GH	PTAG	GH	---	DSKGVG
SPINCLIN2	67	NVLSKLEPNTYD	LDGGKG	---	PTI	GG	---	DSKGVG
SPINCLIN2	68	HO	SPH	KEV	PSFGN	DER	GRN	KQDAEPSS
SPINCLIN1	154	LIDLFHKKIEBALRKL	---	MNSGVC	---	EVYNSVSKL	QYGFGLV	IS
SPINCLIN1	154	LIDLFHKKIEBALRKL	---	MNSGVC	---	EVYNSVSKL	QYGFGLV	IS
SPINCLIN2	154	LIDLFHKKIEBALRKL	---	MNSGVC	---	EVYNSVSKL	QYGFGLV	IS
SPINCLIN2	154	LIDLFHKKIEBALRKL	---	MNSGVC	---	EVYNSVSKL	QYGFGLV	IS
SPINCLIN1	186	KMNQFLYKSHKLVKLV	---	LMV	---	GL	---	---
SPINCLIN1	186	KMNQFLYKSHKLVKLV	---	LMV	---	GL	---	---
SPINCLIN2	186	KMNQFLYKSHKLVKLV	---	LMV	---	GL	---	---
SPINCLIN2	186	KMNQFLYKSHKLVKLV	---	LMV	---	GL	---	---

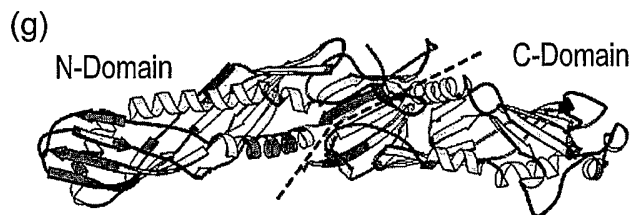
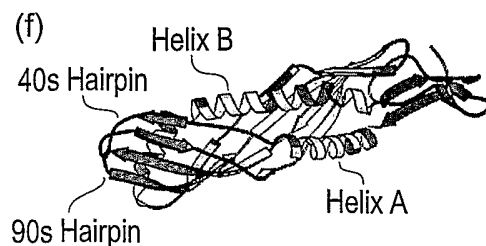


Fig. 12

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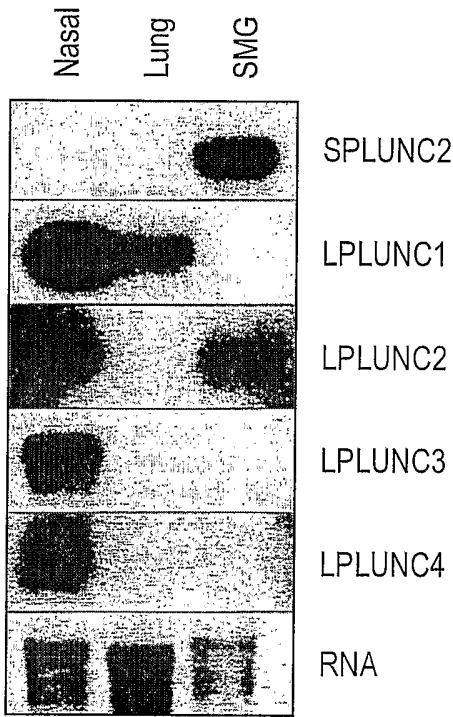


Fig. 13

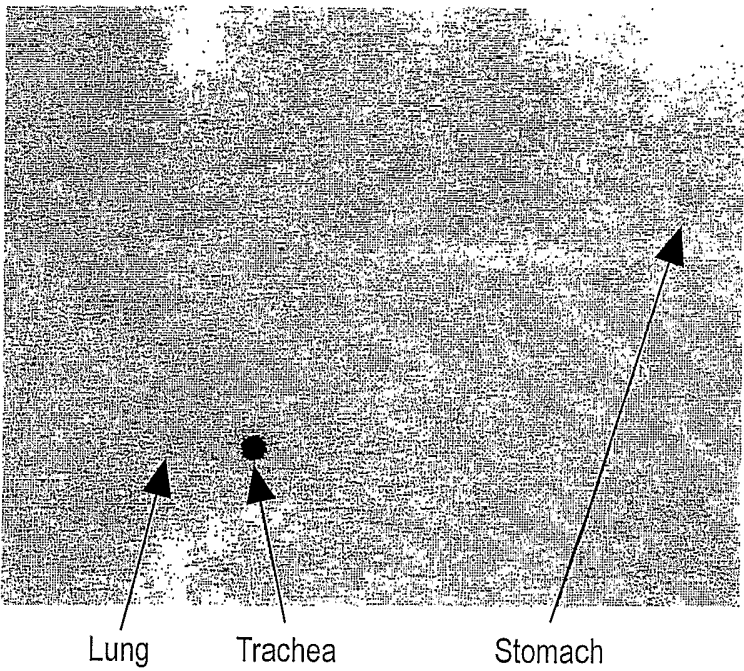
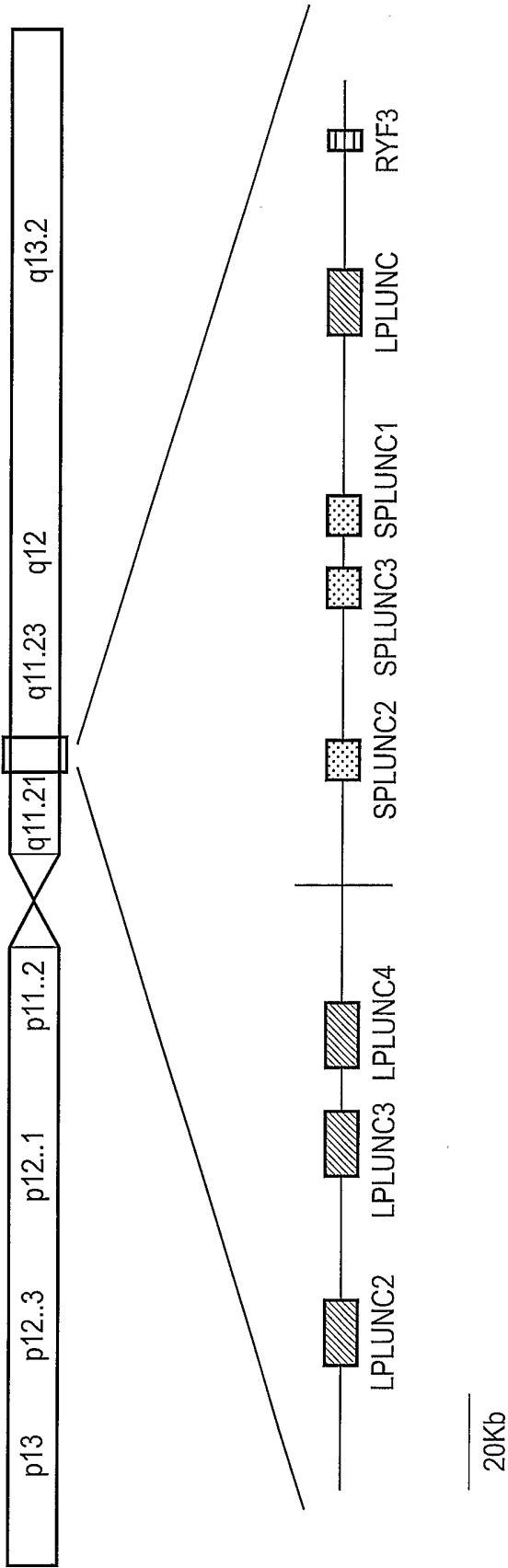


Fig. 14



The human PLUNC gene locus on chromosome 20
Fig. 15

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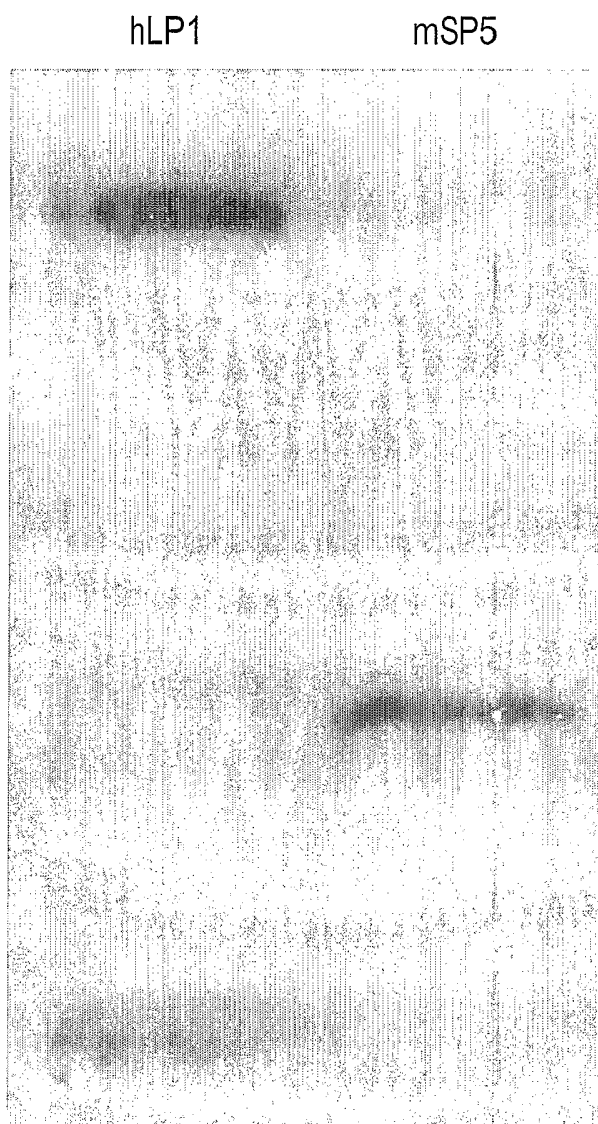


Fig. 16

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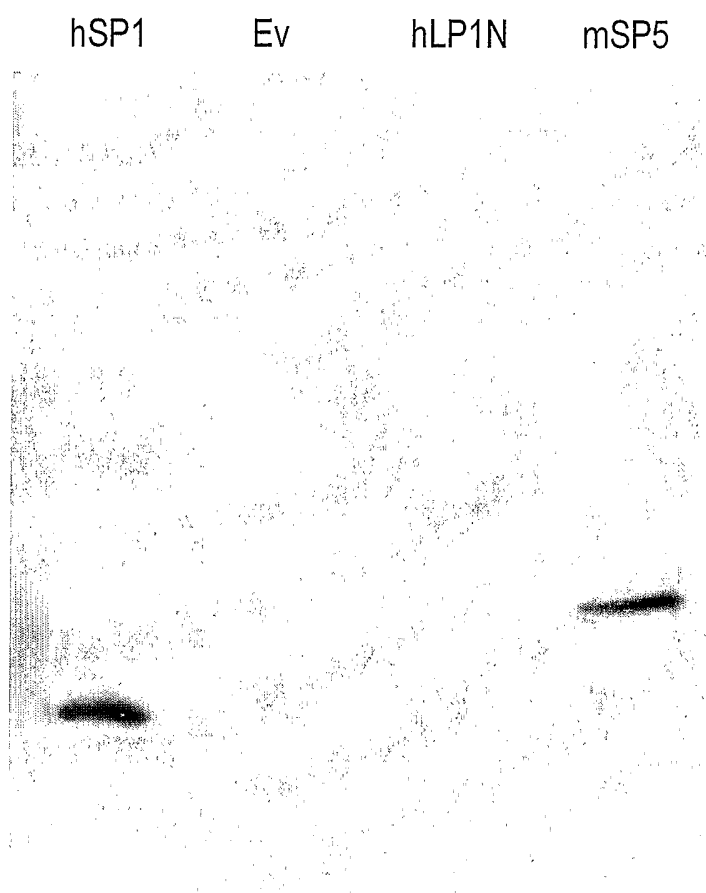


Fig. 17

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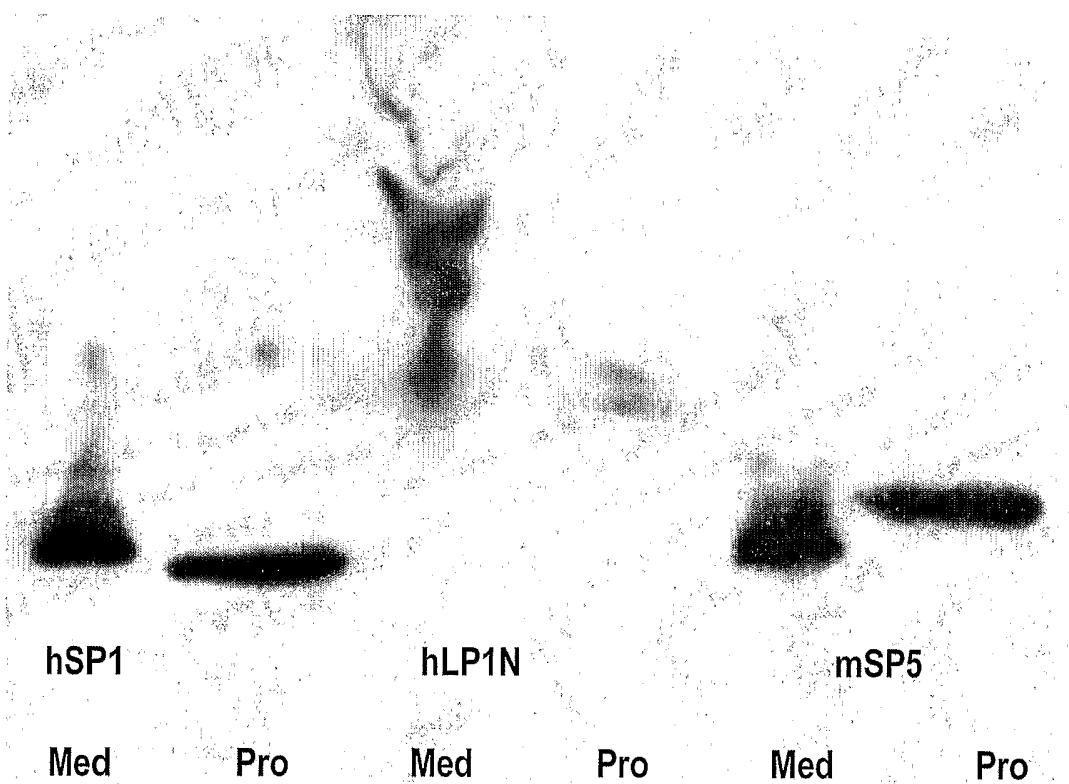


Fig. 18

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Fig. 19

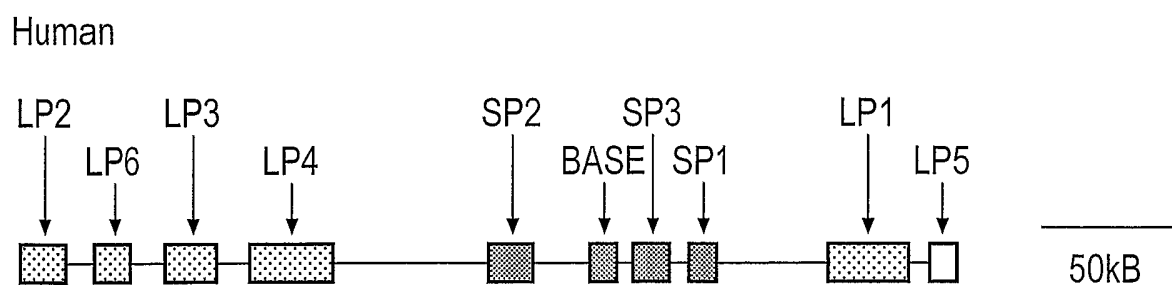


Fig. 20