Antimicrobial Proteins and Peptides

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Introductory article

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Introduction

The production of polypeptides that disrupt microbial structures or interfere with microbial metabolism is a common means of host defence in animals and plants. Although microbial and host structures share many features, antimicrobial polypeptides achieve specificity by targeting components that host cells lack, by exploiting di erences between corresponding host and microbial structures, and by selectively concentrating polypeptides on microbial surfaces. Some host peptides release or activate latent lytic enzymes (autolysins) of their microbial targets, and thereby potentiate their antimicrobial e ects. Specific mechanisms to protect bystander host cells from damage exist, and include cell-associated or soluble macromolecules that bind and detoxify antimicrobial polypeptides.

Infection or injury induces production of some antimicrobial polypeptides. Fruit fly (*Drosophila melanogaster*) mutants unable to induce antimicrobial peptide synthesis succumb to bacterial or fungal infections. Even more direct demonstrations of the importance of antimicrobial polypeptides for host defence have been achieved in plants, where transferring antimicrobial peptide genes from one species to another can produce resistance to specific pathogens.

Mucosal Host Defence

Initial encounters between microbes and multicellular animals often take place on moist surfaces (mucosae). These are coated with glycoproteins (mucins) that interfere with microbial attachment. Epithelia constitute physical barriers to microbial penetration. When ciliated, they mechanically remove microbes by transporting them in a blanket of mucus. Mucosal cells secrete antimicrobial peptides and proteins that damage microbes (**Table 1**). In some organs, such as the lungs, resident host defence macrophages patrol the epithelial surfaces, ingesting microbial trespassers. When surface-adherent microbes multiply or breach the epithelial barrier, a ected host tissues produce signalling molecules (cytokines) that may induce the synthesis of additional antimicrobial polypeptides. Concurrently, various microbial or host-derived molecules trigger an influx of mobile blood cells into the infected area.

Phagocytosis

Phagocytes are specialized host defence cells that can ingest microbes and expose them to antimicrobial substances. Two general types of phagocytes exist: the potentially long-lived macrophages and the short-lived granulocytes. Mature granulocytes are smaller than macrophages, manifest considerably less protein synthesis, and contain abundant cytoplasmic granules that are a repository of antimicrobial proteins and peptides and of hydrolytic enzymes. Phagocytes recognize their targets by phagocytic receptors coupled to the cellular machinery required for motility and ingestion. Certain receptors, such as the mannose receptor on macrophages, bind microbial surfaces directly, while others bind via intermediate adapter molecules (opsonins) deposited onto microbial surfaces from the surrounding fluid. Specific antibodies (especially immunoglobulin G (IgG) class), complement fragments C3b and C3bi, and plasma lectins such as mannose-binding protein are particularly e ective opsonins.

Opsonins bound to microbial surfaces interact with Fcγ receptors, C3b receptors (complement receptor 1, CR1), C3bi receptors (complement receptor 3, CR3), and other phagocytic receptors, causing the plasma membrane of the phagocyte to gradually creep around the target microbe and to form a vacuole called the phagosome. Engagement of phagocytic receptors triggers cytoskeletal rearrangements, and fusion of granules or transport vesicles containing antimicrobial polypeptides to the phagosomal vacuole. Concurrently, NADPH oxidase complexes are

Table 1 Antimicrobial peptides and proteins secreted by mucosal cells

Polypeptide	Mass (kDa)	Distribution
Peroxidases	150	
Myeloperoxidase		Neutrophils, monocytes
Eosinophil peroxidase		Eosinophils
Lactoperoxidase		Epithelia (milk, saliva)
Lactoferrin	80	Neutrophils
		Various epithelia
Bactericidal permeability-increasing protein (BPI)	55–60	Neutrophils
Serprocidins (cathepsin G, elastase, proteinase 3, azurocidin/CAP37)	25–30	Neutrophils
Lysozyme	14	Neutrophils, monocytes, macrophages
		Various epithelia
Phospholipase A_2	14	Neutrophils, macrophages, platelets
		Paneth cells
		Many other tissues
Secretory leucoprotease inhibitor (SLPI)	12	Macrophages
		Various epithelia
Cathelicidins	1–18	Neutrophils (secondary granules)
		Inflamed skin
		Testes
Defensins	4	Neutrophils
		Various epithelia

assembled and activated in the phagosomal membrane, allowing copious production of superoxide anions. These undergo further reactions that generate hydrogen peroxide, and (in concert with myeloperoxidase) more potent antimicrobial oxidants. In some species, a phagocyte enzyme that generates nitric oxide is also induced. The combined action of oxidants and antimicrobial polypeptides destroys most ingested microbes within minutes. The sequestration of microbial targets in phagocytic vacuoles exposes microbes to high concentrations of cytocidal substances while minimizing collateral damage to the host cell.

The most abundant granulocyte in human blood is the polymorphonucleated neutrophil (called PMN, 'poly', or neutrophil, for short). Although the cytoplasmic granules of human neutrophils are heterogeneous, one of its granule subsets (the primary or azurophilic granule) functions to deliver antimicrobial components to the phagosome. Other granule subsets – called secondary (or specific) and tertiary granules – secrete their contents into the extracellular fluid or into the plasma membrane.The primary and secondary granules of human neutrophils both contain antimicrobial polypeptides.

Lysozyme

Lysozyme (also called muramidase) is a widely distributed, cationic (i.e. positively charged) enzyme that cleaves the β 1,4-glycosidic linkage between *N*-acetyl muramate and *N*-acetyl glucosamine. It was first described by Sir Alexander Fleming in 1922, who called attention to its remarkable bacteriolytic properties. Because of its small size, abundance and easy purification, its crystal structure and catalytic mechanism have been extensively investigated. The principal target of lysozyme is peptidoglycan, a structural glycosidic polymer that gives bacteria shape stability and osmotic resistance. Disruption of the peptidoglycan layer su ces to kill some bacteria and renders others more susceptible to osmotic stress. Enzymes with lysozyme activity are found in the digestive organs of many animals and also in host defence settings. Chitinases are functionally and structurally related enzymes that degrade chitin, an exoskeletal polymer of fungi and insects.

High concentrations of lysozyme occur in avian egg white, and in mammalian tears, milk, respiratory secretions, granulocytes and macrophages. In human granulocytes, lysozyme is highly abundant in both primary and secondary granules, and it is a principal secreted protein of macrophages. Organisms susceptible to low concentrations of lysozyme include certain nonpathogenic Gram-positive bacteria (e.g. *Micrococcus lysodeikticus* and *Bacillus* species). Gram-negative bacteria are more resistant because their outer membranes hinder the access of lysozyme to peptidoglycan. At high but still realistic concentrations of lysozyme, nonenzymatic antimicrobial activity that depends on the highly cationic nature of this

molecule is also observed. The abundance and wide distribution of lysozyme suggest that it is of considerable importance.

A single gene on chromosome 12 encodes the human lysozyme enzyme. The mouse has two lysozyme genes, one of which (lysozyme M) is expressed in macrophages and granulocytes, while the other (lysozyme P) is expressed by Paneth cells of the small intestine. In other animals, the number of lysozyme genes and their tissue-specific patterns of expression are highly variable.

Complement

The direct microbicidal e ect of blood plasma on many microbial species is mediated by complement. The key lethal event is the assembly of the membrane attack complex (MAC) consisting of components C5b, C6, C7, C8 with two or more associated C9 molecules in the membranes of target microbes. The insertion of the initiating C5b fragment is the end result of complement activation via one of three complement cascades. The classical pathway is initiated by the binding of C1q molecules to specific antibody clustered on microbial surfaces, the alternative pathway is triggered by the interaction of C3b with the microbial surface, while the lectin pathway depends on contact of C1q-like mannose-binding protein with specific repeating glycans.

The formation of the membrane attack complexes disrupts the ionic homeostasis of the microbes, killing susceptible microbes. Many pathogenic microbes have evolved thick cell walls or capsules that make them partially or completely resistant to complement. The importance of the direct microbicidal activity of complement is illustrated by the predisposition of patients with a deficiency of C6, C7 or C8 to recurrent infections with *Neisseria gonorrhoeae* and *N. meningitidis*. Complement-like proteolytic cascades appeared early in the evolution of multicellular animals and have a documented antimicrobial function already in arthropods (e.g. horseshoe crabs).

Defensins

Defensins are 3–4-kDa cationic antimicrobial peptides with three intramolecular cystine disulfide bonds and a largely β sheet structure. The vertebrate defensins

Bactericidal Permeability-increasing Protein (BPI)

Bactericidal permeability-increasing protein (BPI) is a highly cationic 55–60-kDa protein, abundant in human and other mammalian neutrophils. It is structurally related to the lipopolysaccharide-binding protein (LBP) in plasma, and like LBP, binds to lipopolysaccharide with high a nity. It kills many Gram-negative bacteria by a process that initially involves the permeabilization of their outer membranes, but is inactive against Gram-positive bacteria. BPI is an e ective scavenger of lipopolysaccharide and recombinant BPI has promise as an experimental treatment in meningococcal and other forms of Gram-negative sepsis.

Human BPI is a highly elongated molecule formed by two domains of similar size connected by a proline-rich linker. An N-terminal domain of BPI expresses the bactericidal and endotoxin-neutralizing activities of BPI while the C-terminal domain may be required for binding events that facilitate phagocytosis of BPI-coated bacteria. The human genes encoding BPI, LBP and several other lipid-binding proteins are in close proximity on chromosome 20 (q11.23–q12), suggesting a common ancestral origin. Despite their similarity in overall structural and functional design, the biological activities of BPI and LBP are strikingly di erent: whereas BPI is bactericidal toward Gram-negative bacteria and inhibits endotoxin signalling, LBP does not kill bacteria and greatly enhances the pro-inflammatory action of low doses of LPS.

Initial interactions between BPI and target bacteria probably involve electrostatic attraction between the cationic regions of BPI and the anionic moieties clustered near the highly conserved lipid A region of LPS. In the outer membrane of intact bacteria, the insertion of BPI triggers the rearrangement of outer membrane lipids (LPS and phospholipids) and displacement of LPS-bound Mg^{2+} and Ca^{2+}, resulting in increased permeability to small hydrophobic molecules, increased susceptibility of phospholipids to phospholipase A attack, and bacterial growth inhibition. In a rabbit model of peritoneal inflammation, extracellular BPI secreted by granulocytes into inflammatory exudates confers potent bactericidal activity against many Gram-negative bacteria. Some defensins, cathelicidins and low concentrations of complement potentiate the activity of BPI. Gram-negative bacteria with long lipopolysaccharide chains ('Smooth' colony type) tend to be relatively resistant to BPI.

Proteases

Human and other mammalian granulocytes contain elastase, cathepsin G and proteinase 3, all of which are highly cationic 25–30-kDa serine proteases with antibacterial properties (serprocidins). Azurocidin/CAP37 is another bactericidal, but enzymatically inactive, member of the same protein family. Azurocidin/CAP37, neutrophil elastase and proteinase 3 are encoded by a cluster of genes located at the tip of the short arm of chromosome 19. Cathepsin G gene is located in another cluster of serine protease genes on chromosome 14q11.2. Compared to the active serine proteases, azurocidin/CAP37 contains amino acid substitutions in two residues essential for catalytic activity (serine and histidine). In granulocytes, serprocidins are located in the phagocytic (primary, azurophil) granules. Their collective abundance in human neutrophils is similar to the other major granule proteins (lactoferrin, lysozyme, myeloperoxidase and defensins). The antibacterial properties of serprocidins can often be dissociated from their proteolytic activity, and are competitively inhibited by increasing ionic strength, indicating the importance of charge interactions in their mechanism of action. Although neutrophil elastase is modestly microbicidal, it also converts inactive procathelicidins into highly microbicidal cathelicidins. Transgenic mice lacking neutrophil elastase are susceptible to sepsis and death from intraperitoneal infections with Gram-negative bacteria.

Secretory Phospholipase A_2

Human group II phospholipase A_2 (PLA_2) is a potent 14-kDa enzyme encoded by a gene on chromosome 1p35. The enzyme belongs to a large family of secretory PLA_2 enzymes that share similar structure and catalytic machinery, but di er in their preferred substrates. Human group II phospholipase A_2 preferentially acts on the ester linkages of phospholipids found in bacterial membranes. The antimicrobial activity of this phospholipase is not shared by other phospholipases even though their catalytic mechanisms are very similar. Group II PLA_2 is found in granulocytes, Paneth cells, epithelial secretions, and inflammatory fluids. Against many strains and species of Gram-positive bacteria, purified group II PLA_2 is active at nanomolar concentrations. Bacterial killing by PLA_2 depends on binding to the bacterial surface (Ca^{2+}-independent, presumably to sites in the cell wall), penetration of the cell wall and Ca^{2+}-dependent degradation of membrane phospholipids. Against Gram-negative bacteria, independent antibacterial activity of mammalian group II PLA_2 requires millimolar concentrations and is augmented by complement. Moreover, other host defence substances (e.g. bactericidal permeability-increasing protein, BPI) potentiate the action of nanogram per millilitre amounts of the PLA_2 by disrupting the bacterial outer membrane.

Lactoferrin

Similar to the iron-binding plasma protein, transferrin, and encoded by a gene in the same region of chromosome

3q21–23, lactoferrin is an iron-chelating 80-kDa protein found in the secretory (specific or secondary) granules of neutrophilic granulocytes and in epithelial secretions such as airway fluid, cervical mucus and breast milk. The molecule contains two similar lobes, each of which can bind a ferric cation.

Lactoferrin is bacteriostatic by sequestering essential iron from many bacterial species but other species (e.g. *Neisseria gonorrhoeae*) have developed mechanisms for pilfering iron from lactoferrin. Naturally occurring limited proteolysis of lactoferrin yields a cationic bactericidal peptide, lactoferricin, whose action is not dependent on iron sequestration. Patients with specific-granule deficiency lack lactoferrin in their granulocytes but have normal amounts of lactoferrin in their epithelial secretions. The disorder is complex and several other granulocyte proteins important in host defence are also deficient. The patients su er from recurrent bacterial infections.

Cathelicidins

Cathelicidins are a family of antimicrobial peptides and proteins that contain a conserved $\sim$100 amino acid domain, cathelin, at the N-terminus of the precursor polypeptide. In most but not all cathelicidins, the active moieties are highly variable cationic C-terminal peptides that are cleaved from the precursor during or after secretion. Well-described exceptions include the rabbit cathelicidins p15a and p15b, which are microbicidal in the uncleaved, cathelin-containing form. The single human member of this family, variously named FALL-39, LL-37 or hCAP18, is encoded by a gene on chromosome 3p21.3. The C-terminal 37 amino acid segment (LL-37) is a cysteine-free peptide that readily forms an amphipathic α helix. The bovine bactenecins Bac5 and Bac7 and the porcine PR-39 are examples of proline/arginine-rich peptides with repetitive segments, porcine prophenins contain repetitive arginine/phenylalanine-rich segments, the bovine indolicidin is unusually tryptophan-rich while porcine protegrins are four-cysteine, arginine-rich peptides that resemble defensins and the horseshoe crab peptides, tachyplesins. For several bovine and porcine cathelicidins, neutrophil elastase has been shown to be the activating enzyme that releases cathelicidin from its inactive precursor. Many other cathelicidin precursors contain a conserved consensus sequence for elastase cleavage and are likely to be activated by the same mechanism.

The human cathelicidin (LL-37/hCAP18) has been found in neutrophils, testis and inflamed skin. Multiple members of the cathelicidin family have been identified in bovine neutrophils (indolicidin, Bac5, Bac7, cyclic dodecapeptide and others), in rabbit neutrophils (p15a, p15b and CAP18), and in porcine neutrophils (protegrins, PR-39, prophenins, and others). Cathelicidins have also been reported in mouse, guinea pig, sheep and horse neutrophils. In the pig and rabbit, neutrophil-derived cathelicidins have been shown to function as antimicrobial peptides in inflammatory fluids, either alone (protegrins in pig abscess fluid) or synergistically with bactericidal permeability-increasing protein (p15a and p15b in the rabbit ascitic fluid).

Other Antimicrobial Polypeptides

Secretory leucoprotease inhibitor (SLPI, antileucoprotease) is a 12-kDa cationic protein consisting of two similar subdomains. It is abundant in the secretions of respiratory and reproductive epithelia. The N-terminal domain of SLPI is weakly antibacterial and antifungal whereas its C-terminal domain contains the protease inhibitory activity.

Histatins are a family of 24–38 amino acid, histidine-rich peptides produced in the parotid glands and found in saliva. Their antimicrobial spectrum includes *Candida albicans* and *Streptococcus mutans*.

Calprotectin, a complex of 14- and 8-kDa proteins, is remarkably abundant in the cytoplasm of granulocytes and keratinocytes. It may protect the cytosolic compartment of 'professional phagocytes' by inhibiting the proliferation of any organisms that escape confinement in phagocytic vacuoles. Its ability to exert antifungal and antibacterial activity after release from ruptured granulocytes or keratinocytes is inhibited by zinc.

Further Reading

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