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Structure of a Complex of Human Lactoferrin N-lobe with Pneumococcal Surface Protein A Provides Insight into Microbial Defense Mechanism

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Human lactoferrin, a component of the innate immune system, kills a wide variety of microorganisms including the Gram positive bacteria *Streptococcus pneumoniae*. Pneumococcal surface protein A (PspA) efficiently inhibits this bactericidal action. The crystal structure of a complex of the lactoferrin-binding domain of PspA with the N-lobe of human lactoferrin reveals direct and specific interactions between the negatively charged surface of PspA helices and the highly cationic lactoferricin moiety of lactoferrin. Binding of PspA blocks surface accessibility of this bactericidal peptide preventing it from penetrating the bacterial membrane. Results of site-directed mutagenesis, *in vitro* protein binding assays and isothermal titration calorimetry measurements corroborate that the specific electrostatic interactions observed in the crystal structure represent major associations between PspA and lactoferrin. The structure provides a snapshot of the protective mechanism utilized by pathogens against the host's first line of defense. PspA represents a major virulence factor and a promising vaccine candidate. Insights from the structure of the complex have implications for designing therapeutic strategies for treatment and prevention of pneumococcal diseases that remain a major public health problem worldwide.

Keywords: host–pathogen interaction; immunity; lactoferricin; lactoferrin

Introduction

Survival of a microorganism inside the host depends to a large extent on its ability to avoid, escape or counter the host's defense mechanisms. The host's first line of defense against microbial infection relies on the mechanisms of its innate immunity. As part of the innate immune system the body fluids and organized tissues of animals contain a number of naturally produced antimicrobial agents that kill various microbes or inhibit their growth. A major component of this regimen is a multifunctional iron-binding glycoprotein, lactoferrin (LF), found in milk and other exocrine secretions, which plays an active role in host defense. LF's antimicrobial activity, both bacteriostatic and bactericidal, against various pathogens including bacteria, fungi and viruses has been clearly established.[1–3]

LF is secreted into the mucosal fluids.[4] Therefore, bacteria that colonize mucosal surfaces are exposed to LF and must protect themselves from this antimicrobial agent. Various pathogenic species that live in the mucosal environment have developed different mechanisms for adaptation. For example, Gram negative bacteria of the *Neisseriaceae* family use specific receptor systems for acquiring iron from LF[5,6] while various strains of the Gram positive bacterium *Streptococcus pneumoniae* bind LF *via* a surface protein called pneumococcal surface protein A (PspA).[7,8]

Abbreviations used: LF, lactoferrin; hLF, human lactoferrin; bLF, bovine lactoferrin; rhLF, recombinant human lactoferrin (full length); NLF, recombinant human lactoferrin N-lobe; Lfcn, lactoferricin; LfcnH, human lactoferricin; LfcnB, bovine lactoferricin; PspA, pneumococcal surface protein A; ITC, isothermal titration calorimetry.

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S. pneumoniae is one of the most important pathogens affecting human and is responsible for causing at least million deaths among children worldwide annually†. PspA is an important virulence factor expressed on the surface of all pathogenic strains of *S. pneumoniae*.[9] PspA reduces the killing of pneumococci by LF.[10] The protective role of PspA is apparent from the demonstration of increased susceptibility to LF in pneumococcal strains lacking PspA as compared to the wild-type bacteria.[10] Furthermore, anti-PspA antibodies enhance the bactericidal effect of LF against wild-type pneumococci.[10] Although LFs from various mammalian species are highly homologous in their amino acid sequence, and biochemical and structural properties, PspA exhibits preferential binding to human LF (hLF) as compared to the bovine homolog (bLF).[8] Since *S. pneumoniae* is a human pathogen, the preference of PspA for hLF suggests a functional significance for this protein–protein interaction.

LF is a member of the transferrin family of proteins and, as in other transferrins, the N and C-terminal halves of LF form two homologous lobes referred to as the N and C-lobes. Each lobe contains one iron binding site situated in a deep cleft.[11] The low iron saturation level of naturally occurring LF and its extremely high binding affinity for iron allows it to sequester free iron from body secretions and neutrophils depriving the pathogen of this essential metal, thus giving rise to the bacteiostatic effect.[12–14] However, the bactericidal activity of LF is independent of iron, but for reasons not well understood is restricted only to its iron-free or apo form.[3,10,15] This function of apo-LF presumably results from direct interaction with the bacterial surface.

Since Ellison *et al*. first demonstrated that LF damages the outer membrane of Gram negative bacteria[16] it has been proposed that a highly cationic domain located at the N terminus of LF is responsible for its bactericidal activity.[17] The N terminus of LF from human and other mammals contains positively charged bioactive peptides, collectively known as lactoferricins (Lfcn), that are released in the stomach and mucosal secretions through proteolytic digestion of LF.[18,19] The composition of these peptides is characterized by a relatively large proportion of basic amino acids and a number of hydrophobic residues, especially tryptophan, that render them uniquely suitable for interacting with bacterial lipopolysaccharides and disrupting the membrane structure.[20,21] Subsequent studies demonstrated that lactoferricin peptides, LfcnH and LfcnB, derived from hLF and bLF, respectively, exhibit potent antimicrobial activity against many bacteria, fungi and viruses[22,23] although the exact mechanism of this action remains elusive. Recently we showed that a number of shorter peptides derived from LfcnH possess potent bactericidal activity against pneumococci.[10] The central role of PspA in shielding the pneumococci from the lethal action of hLF is underscored by the observation that PspA, either as a component of the bacterial surface or as a recombinant protein, also protects the bacteria from killing by LfcnH peptides.[10]

PspA is a mutidomain protein which remains anchored to the pneumococcal cell wall through a C-terminal choline binding domain.[24] The primary sequence of PspA shows no significant homology to any other protein in the database except a second choline binding surface protein of *S. pneumoniae*, PspC. The molecular mass of PspA ranges between 67 kDa and 98 kDa in various pneumococcal strains. The N-terminal half of mature PspA is predicted to be entirely α-helical.[25] A lactoferrin-binding region of PspA has been localized within residues 168–288 of this α-helical domain.[8] However, the nature of molecular interactions between PspA and LF remains unknown. Moreover, the PspA binding site on LF is not known. It is also not known if PspA binds to one or both lobes of LF.

Data presented here show that PspA binds to only the N-lobe of hLF. In order to gain a detailed understanding of the molecular interactions that protect *S. pneumoniae* and possibly other pathogens from the bactericidal effect of LF, we have determined the crystal structure of the complex of the LF-binding domain of PspA with the N-lobe of human LF. This is the first structure of LF (or either of its lobes) bound to a protein molecule. The structure of the complex reveals that through a set of specific interactions PspA binds to the lactoferricin domain. Results of this structure analysis contradict a previously proposed model.[26] Using site-directed alanine mutations we confirmed that the protein–protein interactions identified in the crystal structure represent the major association between LF and PspA in solution and that the only binding site for PspA on hLF is located on the lactoferricin domain. The structure provides an insight into the mechanism by which this surface protein of pneumococci protects the bacteria from the host's first line of defense.

Results

In vitro binding of PspA and LF

We cloned, expressed, and purified two truncated PspA fragments and two recombinant versions of human lactoferrin, the N-lobe (NLF) and full length (rhLF), as specified in Materials and Methods. $PspA_1$ represents the complete N-terminal α-helical domain of mature PspA from strain Rx1 (residues 1–288). $PspA_2$ contains the region (residues 168–288) that has been shown to be necessary for binding human LF.[8] NLF consists of amino acid residues

† http://www.who.int/vaccines/en/pneumococcus.shtml