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Proteolytic processing of the streptococcal IgG endopeptidase IdeS modulates the functional properties of the enzyme and results in reduced immunorecognition

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ABSTRACT

The important human gram positive bacterial pathogen *Streptococcus pyogenes* employs various virulence factors to promote inflammation and to facilitate invasive disease progression. In this study we explored the relation of the secreted streptococcal cysteine proteases IdeS and SpeB, and neutrophil (PMN) proteases. We found that SpeB is resistant to proteolytic attack in an inflammatory environment, emphasizing the importance of SpeB for streptococcal pathogenicity, while PMN enzymes and SpeB itself process the IgG degrading endopeptidase IdeS. Processing occurs as NH_2-terminal cleavage of IdeS resulting in reduced immunorecognition of the protease by specific antibodies. While the endopeptidase retains IgG cleaving activity, its ability to suppress the generation of reactive oxygen species is abolished. We suggest that the cleavage of NH_2-terminal peptides by SpeB and/or neutrophil proteases is a mechanism evolved to prevent early inactivation of this important streptococcal virulence factor, albeit at the cost of impaired functionality.

1. Introduction

The Gram-positive bacterium *Streptococcus pyogenes* is a major human bacterial pathogen (Carapetis et al., 2005). *S. pyogenes* causes a wide variety of diseases including uncomplicated infections like pharyngitis, scarlet fever and impetigo, but also invasive, putatively life threatening conditions such as septicemia, necrotizing fasciitis and the streptococcal toxic shock syndrome (Cunningham, 2000). *S. pyogenes* employs multiple molecular mechanisms to counteract host immune responses, including the specific IgG cleaving enzyme IdeS (Von Pawel-Rammingen et al., 2002a, 2012; Vincents et al., 2004). This enzyme is distinct from the well-characterized streptococcal cysteine protease SpeB (Von Pawel-Rammingen and Björck, 2003; Von Pawel-Rammingen, 2012) and mediates streptococcal survival in the presence of opsonizing antibodies (Von Pawel-Rammingen et al., 2002a,b; Lei et al., 2001). Cleavage of the IgG heavy chain occurs in the lower hinge region, a region shown to be essential for effector functions of IgG (Brezski et al., 2009; Radaev and Sun, 2002; Duncan and Winter, 1988). Immunoglobulin cleavage by streptococcal IdeS is a two-step process in which single cleaved IgG is generated as an intermediate product prior to the cleavage of the second heavy chain and the generation of Fab and 1/2 Fc fragments (Ryan et al., 2008; Brezski et al., 2009; Vindebro et al., 2013). Importantly, proteolytic cleavage of one IgG heavy chain is sufficient to inactivate IgG mediator functions (Brezski and Jordan, 2010) and thus, IdeS activity interferes efficiently with IgG mediated recruitment of immune cells and complement factors.

The classical streptococcal cysteine protease SpeB is secreted as a 40 kDa pro-enzyme that is subsequently converted into an active 28 kDa mature cysteine protease by either autocatalytic cleavage or mediated by host proteases (Chen et al., 2003; Doran et al., 1999; Elliott, 1945; Lyon and Caparon, 2003). Regulation of *speB* transcription and SpeB expression is complex and involves transcriptional, post-transcriptional as well as post-translational regulation steps (reviewed by Carroll and Musser, 2011 and references therein). SpeB has inversely been coupled to the transition from confined to systemic infections since down-regulation of SpeB abolishes the SpeB mediated proteolytic degradation of streptococcal virulence factors (Aziz et al., 2004; Sumby et al., 2006; Treviño et al., 2009). Persistence and increased expression of such virulence factors

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has been suggested to facilitate the invasive spread of the bacteria and to result in a more severe disease progression (Cole et al., 2006; Kansal et al., 2000; Chatellier et al., 2000). Inactivation of *spe*B occurs through spontaneous mutations in the CovR/S two-component regulatory system that positively and negatively controls transcription of numerous genes encoding for important streptococcal virulence factors. Thus, mutations in *cov*RS result both in downregulation of *spe*B and the up-regulation of other virulence factors, including IdeS (Aziz et al., 2004; Kansal et al., 2010; Okumura et al., 2013).

During infection polymorphonuclear leukocytes (PMN) are the first line of the immune defense and represent a primary obstacle for pathogenic bacteria. PMNs have been shown to be highly important for the selection of *cov*RS mutant strains during infection (Li et al., 2014). *S. pyogenes* inhibits PMN recruitment through the proteolytic degradation of chemoattractants C5a and IL-8 (Wexler et al., 1985; Edwards et al., 2005) and several streptococcal virulence factors modulate the antimicrobial actions of PMNs to efficiently protect the bacteria from phagocytosis (reviewed in Voyich et al., 2004). Although *S. pyogenes* has means to avoid the antimicrobial actions of PMNs, streptococcal infections are accompanied with an intense inflammatory response and activated PMNs releasing pro-inflammatory mediators, reactive oxygen species, and proteolytic enzymes contribute significantly to a highly proteolytic environment at the infection site. Among proteins discharged from activated PMNs are neutrophil serine proteases elastase, cathepsin G, and proteinase 3 that all play important roles for PMN activity against invading microorganisms. The enzymes are stored in their active form in primary granules and are released upon activation of the PMNs or by fusion of the granules with the phagosome. Interestingly, proteolytic cleavage of IgG by IdeS generates 1/2Fc fragments that have been shown to prime PMNs prior to activation by immunocomplexes (Johansson Söderberg and von Pawel-Rammingen, 2008). "Priming" is defined as PMN activation by a second stimulus resulting in an enhanced rate and extent of ROS production by NADPH oxidase (McPhail et al., 1984; Sheppard et al., 2005). A model was proposed in which primed PMN are activated by immunocomplexes and subsequently discharge at a distance from the bacteria, thereby contributing to inflammatory processes and facilitating the spread of *S. pyogenes* to new infection sites (Johansson Söderberg and von Pawel-Rammingen, 2008).

In this study, we explored the relation of PMN proteases and the secreted streptococcal cysteine proteases IdeS and SpeB in order to investigate aspects of the early stages of an invasive infection, directly after the *cov*RS switch has occurred, when SpeB is present together with other streptococcal virulence factors in this highly proteolytic environment.

We found that SpeB is resistant to proteolytic cleavage by neutrophil proteases, while PMN enzymes and SpeB cleave the NH_2-terminal part of IdeS generating a stable processed form of IdeS. NH_2-terminal cleavage of IdeS did only moderately affect the enzymatic activity of the protease, but abolished the ability of IdeS to inhibit ROS production in PMNs. Notably, NH_2-terminal processing also reduced immunorecognition of IdeS by specific α-IdeS antibodies and we suggest that the specific cleavage of NH_2-terminal peptide sequences can be seen as a molecular defense mechanism preventing IdeS from early recognition and inactivation by the host immune system.

2. Material and methods

2.1. Bacterial strains and growth conditions

The *S. pyogenes* M1 strain 5448 is a patient isolate (Okumura et al., 2013) and was routinely grown in Todd Hewitt broth (TH) (Difco) at 37 °C in 5%CO_2.

2.2. Patient material

Patient serum samples were collected in collaboration with MD Mats Sellin from tonsillitis patients visiting primary health care facilities in Umeå, Sweden during 2012 and 2013. Samples were collected from patients with verified streptococcus positive tonsillitis whom have given informed consent (Ethical approval by the ethical committee in Umeå Dnr 2012-422-32 M, and 2013-285-31 M). Paired acute and convalescent serum samples from patients with invasive streptococcal disease were from seven patients treated for *S. pyogenes* bacteremia at the Clinic for Infectious Diseases, Lund University Hospital, Sweden and have been described in Åkesson et al. (2006).

2.3. Purification of SpeB, IdeS and GST-IdeS30-63

The purification of native IdeS and SpeB has previously been described (Von Pawel-Rammingen et al., 2002a; Persson et al., 2013). Recombinant IdeS was expressed and purified as earlier described (Berggren et al., 2012). For creation of recombinant NH_2-terminal peptide GST-IdeS30-63, plasmid pGEX-IdeS (Von Pawel-Rammingen et al., 2002a) was used as template for site-directed mutagenesis using primer IdeS63 (5′GCAAACTTCACGGATCCTGAAGATG-3′) and a Transformer Site directed Mutagenesis Kit (Clontech) according to the manufacturer's instructions. The mutagenesis procedure introduced a *Bam*HI restriction site that was used for a *Bam*HI cut back creating pGEX IdeS30-63.

2.4. Purification of human polymorphonuclear leukocytes

Human polymorphonuclear leukocytes were isolated from heparinized blood using polymorphprep™ (Nycomed Pharma, Norway) as previously described (Johansson Söderberg et al., 2008). Briefly, whole blood was layered onto polymorphprep medium and centrifuged at 400 g for 30 min. After centrifugation, the neutrophil layer was isolated, washed in PBS and cleared from residual erythrocytes by hypotonic lysis in water. Neutrophils were collected by centrifugation, suspended in 1×PBS supplemented with 1 g/L Human Serum Albumin (Octapharma) and counted using a counting chamber.

2.5. Protein stability assays in neutrophil supernatant and with purified neutrophil proteases

For assaying streptococcal protein stability in neutrophil supernatant, PMNs were stimulated with 100 nM fMLP (Sigma) and 10 μM Cytochalasin B (Sigma) for 30 min at 37 °C. Cells were removed by centrifugation and 0.5 μg recombinant IdeS or 0.5 μg SpeB were added to 200 μL of PMN supernatant for various time periods. For stability assays using purified neutrophil proteases, 1 μg purified protein was incubated with 3 mU cathepsin G, 3 mU neutrophil elastase or 100 ng proteinase 3 in PBS at 37 °C for various periods of time. Protein integrity was analyzed by 16% SDS-PAGE using untreated IdeS or SpeB samples as control. The proteins were visualized by staining with Coomassie Blue (R-250) (USB chemicals).

2.6. Inhibitor screen

To identify the catalytic type of IdeS processing proteases 10 μg native IdeS were incubated with approximately 6×10^6 PMNs in the presence of 4 mM Pefabloc (Sigma), 0.1 mM EDTA, 0.1 mM E-64 (Sigma), 0.1 mM Pepstatin (Sigma), or a 1/50 dilution of complete protease inhibitor cocktail (Roche). The proteins were analyzed by