The surface protease PgtE of Salmonella enterica affects complement activity by proteolytically cleaving C3b, C4b and C5

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Abstract Complement activity in mammalian serum is fundamentally based on three homologous components C3b, C4b and C5. During systemic infection, the gastrointestinal pathogen Salmonella enterica disseminates within host phagocytic cells but also extracellularly. Consequently, systemic Salmonella transiently confronts the complement system. We show here that the surface protease PgtE of S. enterica proteolytically cleaves C3b, C4b and C5 and that the expression of PgtE enhances bacterial resistance to human serum. Degradation of C3b was further enhanced by PgtE-mediated plasminogen activation. © 2007 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

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1. Introduction

The human complement is a complex system of plasma proteins and plays a major role in resistance against microbial infections, as it participates in both innate and specific immunity [1]. Activation of the complement cascade leads to opsonization of foreign microbes, release of chemotactic peptides, and finally to disruption of bacterial cell membranes [2]. Three homologous glycoproteins in the complement system, C3, C4 and C5, play a central role in complement function and interact with numerous other complement components [3]. C3b and C4b, generated from C3 and C4, are important components of convertases that promote activation of the complement cascade. The cleavage fragments of C5 are C5a, which induces migration of phagocytes into the infection site, and C5b, which initiates the formation of the membrane attack complex and bacterial lysis [2].

Salmonella enterica serovar Typhimurium is a common food-borne pathogen that mainly causes gastroenteritis in the small intestine, yet approximately 5% of infected humans develop bacteraemia [4]. Typhimurium infection in mice, on the other hand, resembles human typhoid fever, which is a severe systemic infection caused by S. enterica serovar Typhi [5]. The systemic dissemination of Salmonella from the gastrointestinal tract to the liver and the spleen takes place mainly within infected macrophages and dendritic cells [6]. The survival and growth of Salmonella inside these cells is supported by formation of a specific Salmonella-containing vacuole that avoids fusion with lysosomes [7,8]. Salmonella induces apoptosis of infected macrophages [9], and during the systemic infection of S. enterica serovar Typhi, one-third of the bacterial cells are not associated with phagocytic host cells [10]. This suggests the release of salmonellae from macrophages during infection and, at least transient, extracellular dissemination.

The lipopolysaccharide (LPS) in clinical isolates of Salmonella has long O-antigen oligosaccharide chains. O-antigen is a major determinant in serum resistance of salmonellae [11,1]. However, during growth of S. enterica serovar Typhimurium within mouse macrophages, the LPS structure is altered and the length and the amount of the O-antigen are strongly reduced [12,13]. Therefore, other mechanisms for complement resistance probably are needed after release of Salmonella from macrophages, when it is likely to confront complement attack.

The surface protease PgtE of S. enterica belongs to the omp-\textsuperscript{tfn} family of enterobacterial outer membrane aspartate proteases [14]. PgtE and other omp-\textsuperscript{tfn} require rough LPS to be active but are sterically inhibited by the O-antigen [15,16]. Expression of PgtE is upregulated during growth of Salmonella inside macrophages [12,13] and the bacteria released from macrophages exhibit a strong PgtE-mediated proteolytic activity [13]. PgtE proteolytically activates the mammalian plasma proenzyme plasminogen to plasmin [17], inactivates the main physiological inhibitor of plasmin, \textit{\textsuperscript{2}-antiplasmin [13], and mediates bacterial adhesion to extracellular matrices of human cells [16]. This way, PgtE mediates degradation of extracellular matrix components and generates potent, localized proteolytic activity, which may promote migration of Salmonella across extracellular matrices. PgtE also degrades alpha-helical antimicrobial peptides [18], which may be important during intracellular growth of Salmonella.

The omp-\textsuperscript{tfn} Pla of Yersinia pestis is a close ortholog of PgtE and shares functions with PgtE [16,17,19]. Pla cleaves C3 [20], and we therefore used PgtE for cleavage of complement components and protection against complement-mediated killing of bacteria. Our results suggest that PgtE increases serum resistance of Salmonella by cleaving complement components C3b, C4b and C5.

2. Materials and methods

2.1. Complement components

Purified human complement components C4b, C5, factor H and factor I were obtained from Calbiochem. C4bp and C3 were purified from pooled normal human serum (obtained from healthy laboratory...
personnel) as described previously [21,22] and C3b generated by incubating 10 mg of C3 with 0.1 mg TPCK-treated trypsin (Sigma) for 5 min at room temperature. After inactivation of trypsin by 0.3 mg soybean trypsin inhibitor (Calbiochem) C3b was purified with ion exchange chromatography [23].

2.2. Bacterial strains, plasmids and bacterial cultivation

The bacterial strains used were S. enterica serovar Typhimurium 14028R (a rough derivative of 14028) [24], S. enterica serovar Typhimurium 14028R-1 (pgtE-negative derivative of 14028R) [13], and 14028R-1 and Escherichia coli XL1 (a rough K12-strain; StrataGene) complemented with pMRK3 that codes for PgtE [16], with pMRK3, coding for PgtE D206A which is proteolytically inactive due to a catalytic site substitution [16], or with the vector plasmid pSE380 (Invitrogen). To upregulate expression of PgtE, Salmonella 14028R and 14028R-1 were cultivated in PhoP/Q-inducing medium [13]. PgtE-expression in plasmid-complemented Salmonella 14028R-1, as well as in recombinant E. coli XL1 was induced by 5 μM IPTG [19]. After cultivations, the bacteria were collected by centrifugation and suspended in veronal buffered saline for complement cleavage experiments and in phosphate buffered saline for serum sensitivity experiments.

2.3. Cleavage of complement proteins C3b, C4b and C5

To study the cleavage of complement components, 2.4 μg of complement protein C3b, C4b or C5 was incubated in the presence or absence of 1 × 10⁸ bacterial cells in 60 μl of veronal buffered saline at 37 °C for 4 or 22 h. To study the effect of plasminogen on bacterium-mediated C3b cleavage, 3 μg of C3b and 3 μg of human Glu-plasminogen (American Diagnostica) were incubated with 9 × 10⁵ bacteria in 50 μl of veronal buffered saline for 4 h. As a positive control for the cleavage of complement proteins, C3b was incubated with factor I and factor H (10 μg/ml) or with human plasmin (50 μg/ml) (Fluka) and C4b with factor I (2 μg/ml) and C4bp (1 μg/ml). After incubations, the samples were centrifuged and supernatants were analyzed by reducing 10% SDS–PAGE and immunoblotting. The proteins were probed with a soybean trypsin inhibitor (Calbiochem) C3b was purified with ion exchange chromatography [23].

Complement components C3b, C4b and C5 are crucial for the complement cascade [3]. To investigate the ability of S. enterica to cleave these proteins, the complement proteins were incubated with the bacteria for 4 and 22 h and then analyzed by immunoblotting. Cleavage of C3b and C4b (Fig. 1A) was observed with Salmonella 14028R but not with the pgtE-negative derivative 14028R-1. We used the rough S. enterica 14028R to overcome the steric inhibition of PgtE functions by the O-antigen [13,16]. Supporting the previous studies [13,16], derivatives of the smooth Salmonella 14028 failed to show any proteolytic activity towards C3b or C4b (data not shown). Complementation of 14028R-1 in trans with the plasmid pMRK3, which encodes PgtE, restored degradation of C3b and C4b. The strain 14028R-1(pMRK31), expressing PgtE with a catalytic-residue substitution D206A, as well as 14028R-1(pSE380) carrying the plasmid vector, were inefficient in the cleavage (Fig. 1A).

Degradation of C3b and C4b was also studied with recombinant E. coli. The PgtE-expressing E. coli XL1(pMRK3) efficiently degraded C3b and C4b (Fig. 2) whereas XL1(pMRK31) expressing PgtE D206A and the vector carrying XL1(pSE380) were inactive. XL1(pMRK3) also cleaved intact C3 and C4 (data not shown). Similarly to PgtE-expressing Salmonella,
E. coli XL1(pMRK3) cleaved C3b into 46 kDa, 43 kDa, 40 kDa and 35 kDa fragments (Fig. 2). This fragmentation was partially similar to the one seen with factor I-mediated cleavage of C3b (Fig. 1A). In human plasma, factor I together with its cofactor factor H regulates complement by proteolytically inactivating C3b into 68 kDa, 46 kDa and 43 kDa fragments [25,26]. On the other hand, C4b cleavage mediated by factor I and C4bp or by PgtE was different (Fig. 1A). The pgtE-proficient Salmonella 14028R and 14028R-1(pMRK3), but not the pgtE-negative 14028R-1, also cleaved C5 after 22-h incubation (Fig. 1B), whereas only weak cleavage was detectable after a 4-h incubation (data not shown). Also E. coli XL1(pMRK3) degraded C5 (data not shown). Taken together, these results indicate that Salmonella PgtE proteolytically cleaves C3b, C4b and C5.

3.2. Proteolytic cleavage of C3b is enhanced by PgtE-mediated plasminogen activation

Plasmin is a serine protease that degrades many extracellular matrix components and has also been shown to degrade C3b [27]. Recently, it was found that proteolytic activation of plasminogen to plasmin by staphylokinase of Staphylococcus aureus leads to cleavage of the fluid-phase C3b and decreases the amount of C3b at the staphylococcal surface [28]. As PgtE also activates plasminogen [17] we studied the effect of plasminogen activation on PgtE-mediated C3b cleavage (Fig. 3).

Adding of plasminogen to suspensions containing 14028R cells and C3b led to plasmin formation (data not shown) and to formation of an increased amount of the 46 kDa fragment, as well as production of an additional, c. 38 kDa fragment from C3b (Fig. 3). A 38 kDa fragment was also seen in C3b incubated with plasmin (Fig. 3). Salmonella 14028R-1 was inefficient in C3b cleavage (Fig. 3) and plasminogen activation (data not shown). These results suggest that PgtE-mediated plasminogen activation enhances degradation of C3b.

3.3. Serum resistance is increased in PgtE-expressing bacteria

Serum resistance of Salmonella involves several aspects of the cell surface architecture, i.e., the length and irregularity of the O-antigen [1,29] as well as the cell wall proteins Rck [30], TraT [31] and PagC [32]. To study the role of PgtE in serum resistance, Salmonella 14028R and 14028R-1 cells were cultured in PhoP/Q-inducing conditions and incubated in 2% normal human serum. Both Salmonella strains survived and multiplied during a 4-h incubation (Fig. 4A), however, the pgtE-proficient 14028R grew significantly better than 14028R-1 in the presence of normal human serum. On the other hand, in heat-inactivated serum both strains grew equally well (data not shown). This suggests a positive effect of PgtE on bacterial serum resistance. The PgtE-expressing Salmonella 14028R-l(pMRK3) which was cultivated in Luria

Fig. 2. Proteolytic cleavage of complement components C3b and C4b by recombinant Escherichia coli XL1. Purified C3b (above) and C4b (below) were incubated with E. coli XL1 complemented with the vector plasmid pSE380, pMRK3 encoding PgtE or pMRK31 encoding PgtE D206A for 22 h. The C3b and C4b samples were analyzed by Western blotting using antibodies against C3c and C3d or anti-C4c antibodies. Complement proteins incubated in buffer are also shown. Migration distances of molecular weight markers (in kilodaltons) are indicated on the left. On the right, α, β and γ indicate the polypeptide chains of C3b and C4b. Note the complete cleavage of the α′-chain of C3b and of α′- and β-chains of C4b in samples incubated with E. coli XL1(pMRK3).

Fig. 3. The effect of plasminogen on C3b cleavage by Salmonella enterica. C3b was incubated with S. enterica 14028R and the pgtE-negative derivative 14028R-1 in the presence (+) or absence (−) of plasminogen (Plg). The samples were analyzed by Western blotting using anti-C3c and anti-C3d antibodies. C3b incubated in buffer, with factor I (fI) and factor H (fH), and with plasmin is also shown. The black asterisk indicates the 46 kDa peptide formed by Salmonella 14028R, and the 38 kDa peptide formed in the presence of added Plg is shown by the white asterisk. Migration distances of molecular weight markers (in kilodaltons) are indicated on the left. On the right, α′ and β indicate the polypeptide chains of C3b.

Fig. 4. Serum resistance of PgtE-expressing Salmonella enterica and recombinant Escherichia coli XL1. (A) Survival of Salmonella 14028R and 14028R-1 in 2% normal human serum was determined by viable counting after 4-h incubation. Survival of bacteria in serum was calculated relative to bacterial amount at time point 0 h. The values are means ± S.D. of two experiments done in triplicate (P < 0.01; Student’s two-tailed t-test). Survival of Salmonella 14028R-1 complemented with the PgtE-encoding pMRK3 or the vector plasmid pSE380 in 2% serum (B) and E. coli XL1(pMRK3) and XL1(pSE380) in 0.5% serum (C) was determined after 1-h incubation.
broth containing IPTG to induce *pgtE* survived significantly better in serum than 14028R-1(pSE380) carrying the vector plasmid (Fig. 4B). Likewise, survival of the PgtE-expressing *E. coli* XLI(pMRK3) in 0.5% serum was better than that of XLI(pSE380) (Fig. 4C). These results give further evidence that PgtE has a role in serum resistance of *Salmonella*.

4. Discussion

Our results stress the multifunctional nature of serum resistance in *S. enterica*. Smooth LPS is a major resistance mechanism of *Salmonella* against complement attack [11,1]. After growth within mouse macrophages Typhimurium expresses a short LPS O-antigen [13] which exposes *Salmonella* to complement-mediated killing [11,29]. *Salmonella* is a facultatively intracellular pathogen that multiplies both intracellularly and extracellularly and is continuously released from infected macrophages to infect new ones [33,34]. So, the bacterium is likely to transiently require alternate mechanisms to the O-antigen for protection against complement attack. Activity of PgtE is high in Typhimurium cells released from mouse macrophages, and our results suggest that PgtE compensates for the loss of O-antigen and increases serum resistance of *Salmonella*.

Complement component C3b is an opsonin that induces bacterial uptake by phagocytes [2]. *Salmonella* is able to survive within macrophages and dendritic cells [5], whereas neutrophils do not support the growth of *Salmonella* [35]. Therefore, after release from macrophages it may be important for *Salmonella* to cleave and inactivate C3b molecules to prevent uptake by neutrophils. We found that PgtE cleaves C3b to partially similar fragments as does the C3b-inactivating factor I [25,26]. It is thus likely that PgtE-mediated cleavage also leads to inactivation of C3b. Besides opsonization, the inactivation of both C3b and C4b inhibits the formation of complement activating convertases [2].

PgtE-mediated plasminogen activation and α2-antiplasmin inactivation are thought to be important mechanisms for *Salmonella* to cause local proteolysis which damages tissue barrier in *Salmonella* [13,16]. In this study, we found that PgtE-expressing *E. coli* XLI(pMRK3) in 0.5% serum was better than that of XLI(pSE380) (Fig. 4C). These results give further evidence that PgtE has a role in serum resistance of *Salmonella*.

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**References**


