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RESEARCH ARTICLE

Role of group A *Streptococcus* HtrA in the maturation of SpeB protease

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The serine protease high-temperature requirement A (HtrA) (DegP) of the human pathogen *Streptococcus pyogenes* (group A *Streptococcus*; GAS) is localized to the ExPortal secretory microdomain and is reportedly essential for the maturation of cysteine protease streptococcal pyrogenic exotoxin B (SpeB). Here, we utilize HSC5 (M5 serotype) and the in-frame isogenic mutant HSC5Δ*htrA* to determine whether HtrA contributes to the maturation of other GAS virulence determinants. Mutanolysin cell wall extracts and secreted proteins were arrayed by 2-DE and identified by MALDI-TOF PMF analysis. HSC5Δ*htrA* had elevated levels of cell wall-associated M protein, whilst the supernatant had higher concentrations of M protein fragments and a reduced amount of mature SpeB protease, compared to wild-type (WT). Western blot analysis and protease assays revealed a delay in the maturation of SpeB in the HSC5Δ*htrA* supernatant. HtrA was unable to directly process SpeB zymogen (proSpeB) to the active form *in vitro*. We therefore conclude that HtrA plays an indirect role in the maturation of cysteine protease SpeB.

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Abbreviations: E-64, *N*-[*N*-(L-3-transcarboxyirane-2-carbonyl)-L-leucyl]agmatine; **GAS,** group A *Streptococcus*; **HtrA,** High-temperature requirement A; **proSpeB,** SpeB zymogen; **SpeB,** streptococcal pyrogenic exotoxin B; **WT,** wild-type

1 Introduction

The multifunctional chaperone and serine protease high-temperature requirement A (HtrA) (DegP) is involved in the degradation of misfolded or aggregated proteins within the periplasm of Gram-negative bacteria [1, 2]. Homologues of HtrA have been identified in numerous organisms including Gram-positive bacteria, fungi, yeast, plants and mammals [1]. Although Gram-positive bacteria lack a periplasmic

compartment, a growing body of evidence suggests that HtrA plays an important role in the biology of several Gram-positive species, including *Streptococcus pyogenes* [3], *Streptococcus mutans* [4], *Streptococcus pneumoniae* [5], *Bacillus subtilis* [6], *Staphylococcus aureus* [7], *Lactococcus lactis* [8] and *Listeria monocytogenes* [9]. The accumulation of aberrantly folded or aggregated proteins, or the enhanced sensitivity to thermal, oxidative and osmotic stresses, may account for the reduction in virulence observed in HtrA-deficient mutants [1].

S. pyogenes (group A *Streptococcus*; GAS) is a bacterial pathogen responsible for a wide variety of human diseases, ranging from mild suppurative infections of the skin (impetigo) and throat (pharyngitis), to life-threatening invasive infections such as necrotizing fasciitis and streptococcal toxic shock syndrome [10]. Serious immune sequelae, including acute rheumatic fever and glomerulonephritis, may also develop upon repeated GAS infection [10]. The HtrA family of serine proteases typically contain two C-terminal PDZ (postsynaptic density, disc-large, ZO-1) domains, which are implicated in HtrA oligomerization, substrate recognition and modulation of chaperone and protease activity [11]. However, the HtrA of *S. pyogenes* possesses a single C-terminal PDZ domain. Preliminary studies with a polar insertionally inactivated *htrA* mutant suggested that GAS HtrA is essential for survival at high temperatures, tolerance to reactive oxygen intermediates and full virulence [3]. In a more recent study, a polar insertion into GAS *htrA* also resulted in a temperature-sensitive phenotype [12]. However, this phenotype was also observed following the introduction of a polar insertion immediately downstream from *htrA*, suggesting that a polar effect on the expression of an adjacent gene, rather than loss of HtrA, was responsible for the growth defect. Consistent with this hypothesis, an in-frame *htrA* deletion mutant was neither thermally sensitive nor attenuated in a mouse model of subcutaneous infection [12]. The nonpolar *htrA* mutation, however, did prevent processing of the 40 kDa streptococcal pyrogenic exotoxin B (SpeB) zymogen to the biologically active protease, and elevated the expression of the haemolysin streptolysin S (SLS) [12]. These findings suggest that GAS HtrA is not required for survival at elevated temperatures or virulence, but does play a role in the processing and maturation of secreted virulence factors. The localization of GAS HtrA to the ExPortal secretory microdomain on the streptococcal surface supports this hypothesis [13].

In the present study, we use a nonpolar in-frame HSC5Δ*htrA* mutant to investigate the influence of GAS HtrA on cell wall-associated and secreted virulence factors. We demonstrate that HtrA is not essential for SpeB maturation as previously described [12]; rather the absence of HtrA merely impedes the maturation process. HtrA protease is unable to directly process SpeB zymogen (proSpeB) *in vitro* and, therefore, plays an indirect role in SpeB maturation kinetics.

2 Materials and methods

2.1 Bacterial strains, media and culture conditions

S. pyogenes wild-type (WT) strain HSC5 (M5 serotype) and the isogenic in-frame *htrA* deletion mutant HTR10, henceforth designated HSC5Δ*htrA* in this study, are described elsewhere [12]. In-frame allelic exchange *speB* knockout mutants HSC5Δ*speB* and HSC5Δ*htrA*Δ*speB* were constructed using the temperature-sensitive vector pSpeBΔ*cat*-KO as described previously [14]. All GAS strains were routinely propagated at 37°C on horse blood agar (BioMérieux) or in static liquid cultures of Todd-Hewitt broth (Difco), supplemented with 1% w/v yeast extract (THBY). When necessary, SpeB was inactivated by growth in the presence of cysteine protease inhibitor E-64 (*N*-[*N*-(L-3-transcarboxyirane-2-carbonyl)-L-leucyl]agmatine) (Sigma) at a final concentration of 28 μM. *Escherichia coli* strains were cultured at 37°C on LB agar or in LB broth with agitation at 200 rpm. Where appropriate, *E. coli* strains were grown in the presence of kanamycin (50 μg/mL) and ampicillin (100 μg/mL).

2.2 DNA techniques

S. pyogenes chromosomal DNA was extracted using the DNeasy® tissue kit (Qiagen). Plasmid DNA was purified with the Wizard® Plus SV system (Promega) and transformed into *E. coli* using standard techniques [15]. DNA polymerases, ligases and restriction endonucleases were used in accordance with the manufacturers' recommendations. PCR was performed under standard conditions and the resultant products purified with the Wizard® SV clean-up kit (Promega). Automatic fluorescent DNA sequencing analysis was undertaken using the ABI BigDye® terminator v3.1 cycle sequencing kit (Applied Biosystems) and an ABI 377 DNA sequencer (Applied Biosystems). DNA sequence data was analysed using the ABI Prism® DNA sequencing analysis software and assembled with AutoAssembler™ DNA sequence assembly software (Applied Biosystems).

2.3 Expression plasmid construction and protein purification

The HtrA protein of *S. pyogenes* contains a putative N-terminal membrane anchor domain (isoleucine 13 to isoleucine 26), a trypsin-like serine protease catalytic triad (histidine 129, aspartic acid 158 and serine 240) and a single C-terminal PDZ domain (glycine 296 to arginine 385) [3]. The *htrA* gene of GAS strain NS931 (M69 serotype) [16] was PCR amplified with *Taq* DNA polymerase (Qiagen) using the following temperature cycling parameters: 94°C for 2 min; 35 cycles of 94°C for 40 s, 55°C for 40 sec, 72°C for 2 min; 72°C for 4 min; and a holding temperature of 25°C. The forward primer 5′-GGGGATCCACATTCAATAATCTCTACCCA-3′ (*Bam*HI restriction site underlined) and reverse primer 5′-GGAAGCTTTTACTGCGTTTTAGTCAAATC-3′ (*Hin*dIII

restriction site underlined) were designed from the SF370 (M1 serotype) *htrA* sequence [17] located in the NCBI database (GenBank accession number NC_002737.1; http://www.ncbi.nlm.nih.gov). The first 78 nucleotides of GAS *htrA*, which encode the putative transmembrane domain [3], were not amplified. The purified PCR product was digested with the appropriate restriction enzymes, ligated into 6 × His-tag expression vector pQE-9 (Qiagen) with T4 DNA ligase (Fermentas) and transformed into *E. coli* M15 harbouring the pREP4 repressor plasmid (Qiagen). The insert DNA was sequenced (GenBank accession number DQ230906) and identified by a BLAST-N search at NCBI (http://www.ncbi.nlm.nih.gov/BLAST). The translated amino acid sequence shares 99% identity with all putative HtrA proteins published to date for *S. pyogenes* (data not shown), suggesting that this protein is highly conserved amongst GAS isolates. Recombinant 6 × His-tagged HtrA protein was expressed and purified essentially as previously described [18].

The *ropA* gene of *S. pyogenes* encodes for trigger factor (also known as RopA), a ribosome-associated chaperone and peptidyl–prolyl *cis–trans* isomerase (PPIase) implicated in SpeB protease maturation [19]. The *ropA* gene of GAS strain 5448 (M1T1 serotype) [20] was PCR amplified with PfuUltra™ High-Fidelity DNA Polymerase (Stratagene) using the following temperature cycling regime: 95°C for 2 min; 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 2 min 30 s; 72°C for 10 min; and a holding temperature of 25°C. The forward primer 5′-CACCATGTCTACATCATTTGAA-3′ (TOPO® cloning overhang underlined) and reverse primer 5′-TTACTTAACGCTTGCTGTGCT-3′ were designed from the *ropA* sequence of *S. pyogenes* strain MGAS315 (M3 serotype; GenBank accession number AE014074.1) [21] located in the NCBI database (http://www.ncbi.nlm.nih.gov). The blunt-end PCR product was cloned into pET160/GW/D-TOPO® in accordance with the Champion™ pET160 Directional TOPO Expression Kit (Invitrogen). The insert DNA was sequenced and identified by a BLAST-N search at NCBI (http://www.ncbi.nlm.nih.gov/BLAST). Recombinant 6 × His-tagged trigger factor was expressed and purified essentially as described before [18].

2.4 Immunological reagents

The production of polyclonal mouse antiserum specific for *S. pyogenes* M1 protein is described elsewhere [22]. Affinity-purified rabbit anti-SpeB IgG was purchased from Toxin Technology (Sarasota, FL, USA).

2.5 Western blot analysis

SDS-PAGE and Western blotting was performed as described before [23]. The NC membranes were incubated for 1 h with SpeB antibodies diluted 1:1000 in PBS (pH 7.4). Following a 10 min wash with PBS, the membranes were incubated for 1 h with goat antirabbit IgG HRP conjugate (BioRad) diluted 1:1000 in PBS. Excess secondary antibody was removed by three 10 min washes in PBS prior to development in a solution of 100 mM Tris-HCl (pH 7.6) containing 1.4 mM diaminobenzidine and 0.06% v/v hydrogen peroxide. 2-D Western blotting was undertaken using the semi-dry Hoefer SemiPhor™ transfer unit (GE Healthcare) in accordance with the manufacturer's directions.

2.6 Preparation of cell wall extracts and culture supernatant proteins

Mutanolysin cell wall extracts were prepared in duplicate from stationary phase cultures of *S. pyogenes* as described previously [24]. Briefly, 100 mL of cultures were grown for 16 h at 37°C without shaking. Bacterial cells were pelleted by centrifugation at 7560 × *g* for 20 min at 4°C, washed twice with 5 mL of chilled TE buffer (50 mM Tris-HCl, 1 mM EDTA, 1 mM PMSF; pH 8.0) and resuspended in 1 mL of cold TE-sucrose (50 mM Tris-HCl, 1 mM EDTA, 20% w/v sucrose; pH 8.0) supplemented with 100 μL of lysozyme (100 mg/mL in TE-sucrose) and 50 μL of mutanolysin (5000 U/mL in 0.1 M K_2HPO_4; pH 6.2). The sample was incubated for 2 h at 37°C with shaking (200 rpm) and centrifuged at 16 000 × *g* for 5 min at room temperature. The supernatant, enriched with the cell wall-associated proteins, was harvested by aspiration and the protein concentration determined using the Bradford assay (Sigma). Samples for 2-DE were precipitated with an equal volume of 10% v/v TCA [25], and resuspended in standard sample solubilization (SSS) buffer containing 8 M urea, 100 mM DTT, 4% w/v CHAPS (GE Healthcare), 0.8% v/v carrier ampholytes (BioRad) and 40 mM Tris-HCl. Identical amounts of total protein from HSC5 and HSC5Δ*htrA* cell wall extracts were loaded onto 2-D gels.

For the preparation of culture supernatant proteins, GAS strains were cultured in duplicate to stationary phase in protein-reduced THBY, which consisted of THBY filtered through an Amicon® Ultra-15 centrifugation unit (NMWL 10 kDa; Millipore) to remove proteins with a molecular mass greater than 10 kDa. Identical volumes of culture supernatant were harvested by centrifugation, filter-sterilized through a 0.22 μm filter (Millipore), precipitated with an equal volume of 10% v/v TCA and resuspended in 100 mM Tris-HCl (pH 7.6), prior to SDS-PAGE analysis and determination of protein concentration with the Bradford assay (Sigma). Supernatant samples for 2-DE were resuspended in SSS buffer as described above. Identical amounts of total protein from HSC5 and HSC5Δ*htrA* culture supernatants were loaded onto 2-D gels.

2.7 2-DE and protein identification

TCA precipitated cell wall extract or culture supernatant (180 μg) was added to an Immobiline™ DryStrip Reswelling Tray (GE Healthcare) and overlaid with an 11 cm linear pH 4–7 IPG ReadyStrip™ (BioRad). After overlaying with

mineral oil (BioRad), the strips were rehydrated overnight at room temperature. First dimension IEF was performed with the Protean® IEF Cell (BioRad) as described by Cole *et al.* [24]. The second dimension (SDS-PAGE) was conducted using the Ettan™ DALT*six* electrophoresis unit (GE Healthcare) as described previously [24]. Duplicate 2-D gels were generated for each cell wall and culture supernatant sample to verify reproducibility. Differentially expressed proteins were excised from 2-D Coomassie stained gels, digested with trypsin and analysed by MALDI-TOF MS as described elsewhere [24]. Peak lists were manually generated from spectra internally calibrated with peptides derived from trypsin autodigestion. Peptide masses were matched by searching the databases at PeptIdent (http://us.expasy.org/tools/peptident.html) or MASCOT Server 2.0 software (http://www.matrixscience.com), using the search parameters described by Cordwell *et al.* [26].

2.8 Measurement of protease activity

SpeB protease activity in GAS culture supernatants was determined essentially as described before [23, 27]. For *in vitro* protease assays, 5 μg of HtrA or SpeB protease was mixed with 5 μg of purified substrate protein (proSpeB), adjusted to a final volume of 25 μL with PBS (pH 7.4) and incubated at 37°C for 3 h or overnight. Cleavage of substrate protein was determined by SDS-PAGE. Positive controls containing 5 μg of protease (HtrA or SpeB) and 25 μg of β-casein (Sigma) substrate were used in all assays. Negative controls containing only protease or substrate were also included. Purified SpeB protease was purchased from Toxin Technology.

2.9 Statistical analysis

The SpeB protease activity of WT and HSC5Δ*htrA* was compared using the two-tailed unpaired *t*-test. Differences were considered statistically significant at $p<0.05$. All statistical tests were performed using GraphPad Prism version 4.02 software (GraphPad Software, San Diego, CA, USA).

3 Results

3.1 2-DE analysis of cell wall and culture supernatant proteins

Mutanolysin cell wall extracts harvested from stationary phase cultures of WT and HSC5Δ*htrA* were separated by 2-DE (Fig. 1A). Twelve differences were identified by either MALDI-TOF PMF or 2-D Western blotting (Table 1). M protein, a major surface-associated and secreted GAS virulence factor [10], was only detected in the HSC5Δ*htrA* cell wall (spot 6). Seven differences in the stationary-phase secreted proteomes (Fig. 1B) were identified by PMF (Table 2). Fragments of M protein (spots 21–24; Fig. 1B) were only detected in the HSC5Δ*htrA* culture supernatant. Compared to WT, the HSC5Δ*htrA* supernatant contained a high concentration of proSpeB (spot 19) and a reduced amount of mature SpeB protease (spot 16; Fig. 1B), a secreted virulence factor with a major role in GAS pathogenesis [23, 28]. In contrast with previous work [12], this finding suggests that HSC5Δ*htrA* can produce active SpeB, although less efficiently than the WT. Given that SpeB is responsible for the degradation of many proteins in the secreted GAS proteome [20], the reduced concentration of active SpeB in HSC5Δ*htrA* may account for the incomplete degradation of surface-displayed and secreted virulence factors. Western blot analyses of cell wall and culture supernatant proteins revealed a reduction in the degradation rate of M protein for HSC5Δ*htrA* (Fig. 1C). Equivalent quantities of M protein were detected in the cell wall and supernatant of HSC5Δ*speB* and HSC5Δ*htrA*Δ*speB*, suggesting that the reduced rate of M protein degradation in HSC5Δ*htrA* is due to reduced or delayed SpeB activity.

3.2 Role of HtrA in SpeB maturation kinetics

To establish whether the absence of *S. pyogenes* HtrA kinetically delays the conversion of proSpeB to active SpeB protease, Western blot analysis of WT and HSC5Δ*htrA* culture supernatant harvested at 7, 10, 16 and 26 h postinoculation (Fig. 2A) was undertaken with SpeB-specific antisera (Fig. 2B). At 7 h postinoculation, 40 kDa proSpeB is detected in WT and HSC5Δ*htrA* supernatant, with levels elevated in HSC5Δ*htrA* (Fig. 2B). After 10 h, the concentration of proSpeB is greater for HSC5Δ*htrA* and 28 kDa SpeB is only present in the WT supernatant (Fig. 2B). The majority of WT proSpeB has been converted to SpeB at 16 h in contrast to HSC5Δ*htrA*, where only a relatively small amount of mature SpeB is observed. However, at 26 h postinoculation, similar levels of mature SpeB protease are present in both strains, although the concentration of proSpeB remains higher for HSC5Δ*htrA* (Fig. 2B). Culture supernatants from parallel 16 h cultures of HSC5Δ*speB* and HSC5Δ*htrA*Δ*speB* (Fig. 2A) were negative for SpeB expression (Fig. 2B). With the addition of SpeB inhibitor E-64 to the growth medium, WT and HSC5Δ*htrA* secrete equivalent quantities of proSpeB into the extracellular milieu (data not shown). These data suggest that loss of HtrA does not affect proSpeB secretion; rather the lack of HtrA may delay the conversion of proSpeB to active SpeB in the supernatant. The delayed SpeB activity of HSC5Δ*htrA* may reduce the SpeB-mediated degradation rate of cell wall-associated and secreted proteins, which may account for the increased number of protein spots detected in the proteomic analyses of HSC5Δ*htrA*, in comparison to WT (Fig. 1).

3.3 Quantitative time-course analysis of SpeB activity

In vitro SpeB assays confirm that neither WT nor HSC5Δ*htrA* secrete active SpeB protease into the supernatant 7 h postinoculation (Fig. 3). At 10 h after inoculation,

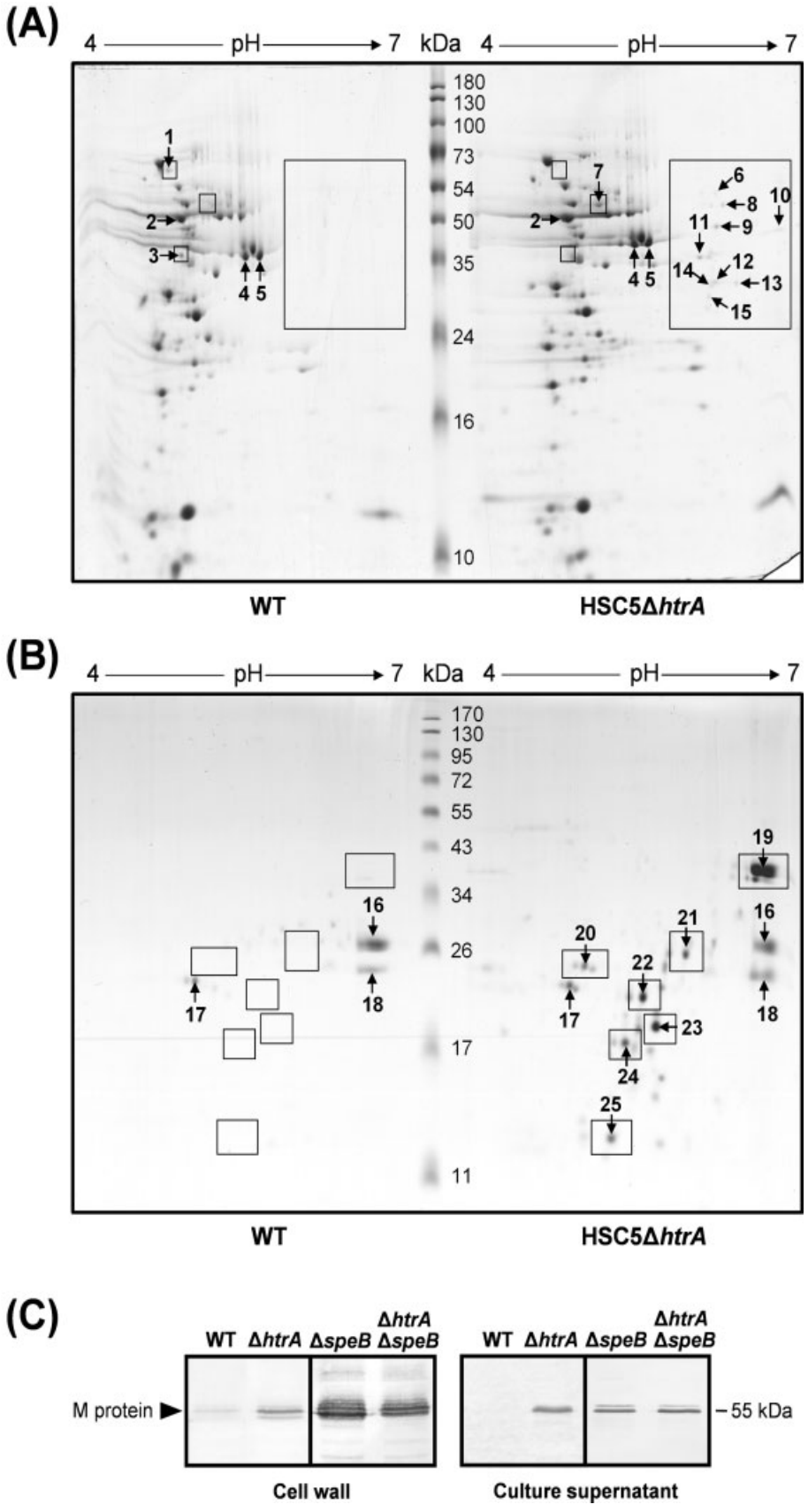

Figure 1. Comparison of cell wall and secreted proteomes of HSC5 (WT) and HSC5Δ*htrA*. Differences (boxed) were identified by MALDI-TOF PMF or Western blot analyses. (A) 2-DE of WT and HSC5Δ*htrA* cell wall extracts harvested after growth to stationary phase (16 h at 37°C) in THBY medium without agitation. Identified protein spots are denoted by numbered arrows, which correspond to the proteins in Table 1. (B) 2-DE of WT and HSC5Δ*htrA* culture supernatants harvested after growth to stationary phase (16 h at 37°C) in protein-reduced THBY medium without agitation. Identified protein spots are denoted by numbered arrows, which correspond to the proteins in Table 2. (C) Detection of M protein in stationary phase (16 h) cell wall and culture supernatant proteins of WT, HSC5Δ*htrA*, HSC5Δ*speB* and HSC5Δ*htrA*Δ*speB* by Western blot analyses with M protein-specific antiserum. Molecular mass markers are given in kilo-Daltons (kDa).

Table 1. Cell wall-associated proteins identified by MALDI-TOF PMF or 2-D Western blotting from stationary phase cultures of HSC5 (WT) and HSC5Δ*htrA*

Spot	Protein	Function or pathway	Accession no.[a]	Molecular mass (kDa)[b]	p*I*[b]	Peptide match[c]	Coverage (%)[d]	GAS strain[e]	
								WT	HSC5Δ*htrA*
1	Chaperone protein DnaK (HSP70)	Chaperone	AAM80138	64.8	4.61	15	30.5	+	
2	Enolase (SEN)	Virulence factor	AAM79086	47.2	4.74	23	67.3	+	+
3	Chaperone protein DnaK (HSP70) fragment	Chaperone	AAM80138	35.5	4.81	17	24.0	+	
4	GAPDH	Virulence factor	AAT86400	35.8	5.34	19	54.9	+	+
5	GAPDH	Virulence factor	AAT86400	35.8	5.34	20	60.6	+	+
6	M protein[f]	Virulence factor							+
7	Elongation factor Tu	Protein biosynthesis	AAM79039	43.8	4.91	18	56.5		+
8	Putative glutathione reductase	Glutathione metabolism	AAK33749	48.9	5.75	10	26.0		+
9	Putative maltose/malto-dextrin-binding protein	Sugar transporter activity	AAK34142	44.6	7.57	13	36.0		+
10	SpeB precursor	Virulence factor	AAK34706	43.2	8.76	6	18.0		+
11	Glycerol-3-phosphate dehydrogenase [NAD(P)+]	Phospholipid biosynthesis	AAM78768	36.7	5.65	16	55.9		+
12	Two-component response regulator YesN	Signal transduction	AAT87437	28.1	5.05	5	30.0		+
13	Malonyl-CoA-[acyl-carrier-protein] transacylase	Fatty acid biosynthesis	AAT87620	34.5	6.25	11	39.8		+
14	Putative lipoprotein	Lipid binding	BAC64163	36.4	7.60	5	14.0		+
15	Putative L-ribulose 5-phosphate 4-epimerase	Metabolism	AAM78747	26.1	5.85	7	31.0		+

a) GenBank accession number.
b) Theoretical values.
c) Number of tryptic peptides matched to the protein.
d) Percentage of protein sequence covered by the matched peptides.
e) Proteins identified in WT and/or HSC5Δ*htrA* are denoted by a plus sign.
f) Identified by 2-D Western blot analysis.

the WT exhibits 20-fold more SpeB protease activity compared to HSC5Δ*htrA* (Fig. 3). Equivalent SpeB activity is observed for both strains at 16 h postinoculation, whilst at 26 h significantly more activity is detected in the HSC5Δ*htrA* supernatant ($p<0.05$; Fig. 3). Supernatants harvested from HSC5Δ*speB* and HSC5Δ*htrA*Δ*speB* were included as negative controls for SpeB activity (Fig. 3). Assays performed in parallel with SpeB inhibitor E-64 also exhibited no SpeB activity (data not shown), confirming that all protease activity in the culture supernatants can be attributed to SpeB. These results demonstrate that a lack of *S. pyogenes* HtrA delays the maturation of proSpeB to active SpeB.

3.4 Role of HtrA in proSpeB activation

To ascertain whether HtrA protease acts directly on proSpeB in the GAS supernatant *in vitro*, protease assays were performed using recombinant 6×His-tagged GAS HtrA and proSpeB isolated from GAS culture supernatants. The degradation of β-casein substrate confirms that recombinant HtrA is proteolytically active (Fig. 4A). In many organisms, HtrA plays a role in the degradation of unfolded or misfolded proteins destined for secretion across the cytoplasmic membrane [1]. However, GAS HtrA was unable to degrade proSpeB in the presence or absence of denaturants such as 6 and 8 M urea (Fig. 4A). In addition, E-64 and urea did not inhibit the proteolytic activity of HtrA, as demonstrated by the complete degradation of β-casein *in vitro* (data not shown). Western blot analysis confirms that HtrA did not directly process the proSpeB (Fig. 4B). These data suggest that *S. pyogenes* HtrA is not directly involved in SpeB maturation *in vitro*. Consistent with prior studies [29], active SpeB protease processed proSpeB (Fig. 4C). In the presence of E-64, however, SpeB did not fully degrade β-casein or activate proSpeB, indicating that E-64 is an effective inhibitor of SpeB activity. Trigger factor (RopA), a PPIase implicated in SpeB maturation [19, 30], was unable to directly process proSpeB to mature SpeB *in vitro* in the absence (Fig. 4C) or the presence of HtrA (data not shown).

Table 2. Culture supernatant proteins identified by MALDI-TOF PMF from stationary phase cultures of HSC5 (WT) and HSC5Δ*htrA*

Spot	Protein	Function or pathway	Accession no.[a]	Molecular mass (kDa)[b]	p*I*[b]	Peptide match[c]	Coverage (%)[d]	GAS strain[e]	
								WT	HSC5Δ*htrA*
16	SpeB	Virulence factor	BPSOP	36.7	5.84	10	42.0	+	+
17	Streptodornase 1 (Sda1)	Virulence factor; DNase activity	AAZ51787	30.1	5.01	3	16.0	+	+
18	Streptodornase B (SdaB) (fragment)	Virulence factor; DNase activity	BAD74233	25.7	8.61	4	25.0	+	+
19	SpeB precursor (fragment)	Virulence factor	1DKIA	33.4	7.22	10	42.0		+
20	Hypothetical membrane spanning protein	Unknown	ABF33010	34.8	9.85	3	9.0		+
21	M protein (fragment)	Virulence factor	AAQ73237	27.3	6.04	11	37.0		+
22	M protein precursor (fragment)	Virulence factor	AAN46651	23.2	5.57	9	35.0		+
23	M protein precursor (fragment)	Virulence factor	AAN46651	23.2	5.57	9	36.0		+
24	M protein precursor (fragment)	Virulence factor	AAN46651	23.2	5.57	6	23.0		+
25	CoA-binding protein	Acyl-coenzyme A binding	ABF33227	16.8	6.59	2	21.0		+

a) GenBank accession number.
b) Theoretical values.
c) Number of tryptic peptides matched to the protein.
d) Percentage of protein sequence covered by the matched peptides.
e) Proteins identified in WT and/or HSC5Δ*htrA* are denoted by a plus sign.

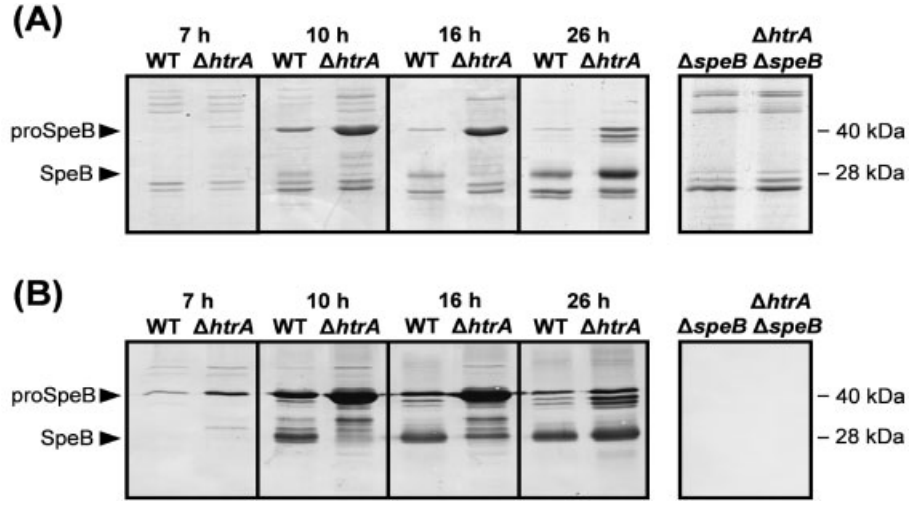

Figure 2. *S. pyogenes* HtrA is indispensable for the efficient conversion of proSpeB to the active form. (A) Coomassie stained 12% SDS-PAGE reducing gel and (B) corresponding SpeB Western blot of HSC5 (WT) and HSC5Δ*htrA* culture supernatants collected after growth at 37°C for 7, 10, 16 and 26 h in protein-reduced THBY medium without aeration. Samples were concentrated by TCA precipitation prior to transfer to NC membrane and incubation with antiserum specific for SpeB. The 40 kDa proSpeB and the 28 kDa mature SpeB protease are indicated by labelled arrow heads. Supernatants harvested from 16 h cultures of HSC5Δ*speB* and HSC5Δ*htrA*Δ*speB* were included as negative controls for SDS-PAGE and Western blot analyses. Molecular mass markers are given in kDa.

4 Discussion

A growing body of data indicates that HtrA influences the processing of surface-associated proteins in Gram-positive bacteria. The HtrA homologue of *L. lactis*, termed $HtrA_{Ll}$, is a unique surface housekeeping protease responsible for the pro-peptide processing and maturation of secreted zymogens, including nuclease NucA and autolysin AcmA [31]. In *S. mutans*, the causative agent of dental caries, HtrA plays a role in the regulation of genetic competence, biofilm forma-

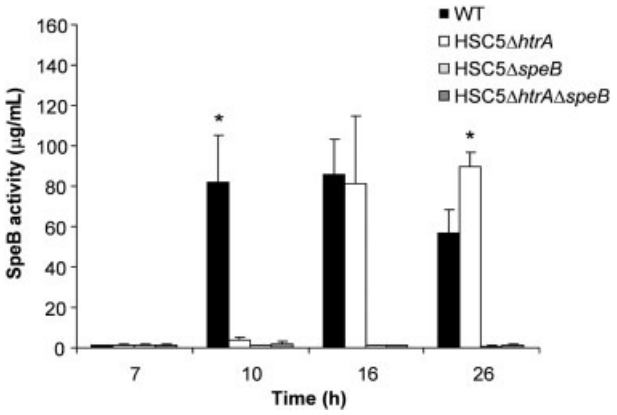

Figure 3. The absence of HtrA slows the conversion of proSpeB to the mature protease. *In vitro* SpeB activity of culture supernatant harvested from HSC5 (WT) and HSC5Δ*htrA* at 7, 10, 16 and 26 h postinoculation. HSC5Δ*speB* and HSC5Δ*htrA*Δ*speB* supernatants were included as SpeB negative controls. All strains were grown at 37°C in protein-reduced THBY medium without shaking. The values represent the mean and SEM of four independent experiments. Asterisks indicate a statistically significant difference between WT and HSC5Δ*htrA* ($p<0.05$).

tion and the biogenesis of cell wall-associated and secreted proteins [4, 32]. *B. subtilis* expresses three HtrA-like proteases including HtrA/YkdA, HtrB/YvtA and YyxA [33, 34], which are predicted to be membrane bound. Under conditions of heat shock or secretion stress, the HtrA and HtrB proteases of *B. subtilis* are essential for the degradation of misfolded proteins and the secretion of correctly folded proteins [35]. The human pathogen *S. aureus* expresses two surface-exposed HtrA homologues designated $HtrA_1$ and $HtrA_2$. These proteins are involved in the secretion of virulence factors essential for bacterial dissemination and may play a role in the maturation of the *agr* surface components [7].

In *S. pyogenes*, proteins are exported *via* the general secretory (Sec) pathway from multiple different subcellular regions [36, 37]. SpeB is secreted from the ExPortal, a distinct cellular membrane microdomain [37] enriched with anionic phospholipids [38] to facilitate protein translocation. GAS HtrA is localized to the ExPortal, which suggests a role for HtrA in the elaboration of secretory proteins [13]. In this study, we have demonstrated that GAS HtrA is not required for SpeB maturation as previously suggested [12]. Furthermore, HtrA does not play a direct role in SpeB processing *in*

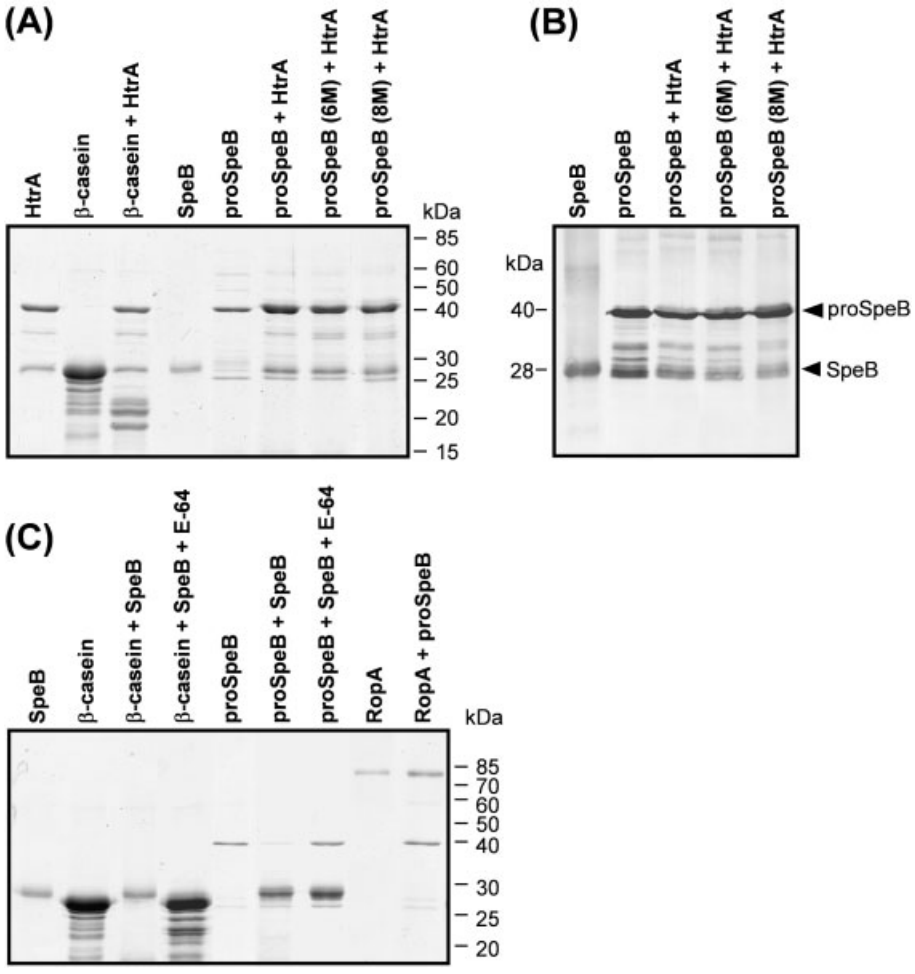

Figure 4. HtrA protease does not directly process proSpeB. (A) Coomassie stained 12% SDS-PAGE reducing gel showing *in vitro* protease assays of recombinant 6×His-tagged HtrA with β-casein (positive control), proSpeB and proSpeB denatured in 6 or 8 M urea. HtrA protease retains proteolytic activity in the presence of urea (data not shown). (B) Corresponding Western blot of HtrA and proSpeB protease assays probed with anti-SpeB antisera. (C) Coomassie stained 12% SDS-PAGE reducing gel showing *in vitro* protease assays of SpeB with β-casein (± E-64), SpeB with proSpeB (± E-64) and trigger factor (RopA) with proSpeB. Molecular mass markers are given in kDa.

vitro. However, the absence of HtrA does dramatically slow the maturation kinetics of SpeB, which in turn reduces the rate at which SpeB protease cleaves surface-associated and secreted GAS virulence factors.

SpeB is a highly conserved extracellular and cell surface-associated protease expressed by most GAS isolates [39]. The cysteine protease cleaves many biologically important molecules and plays a significant role in GAS pathogenesis [23, 28]. The expression of SpeB is growth-phase dependent, with the maximal rate of expression occurring from late logarithmic to stationary phase in response to nutrient and carbon source limitation, acidic pH and NaCl concentration [40]. SpeB is initially synthesized as a 43 kDa pre–pro-protein with a N-terminal signal sequence, followed by a pro-sequence region, and a mature cysteine protease sequence. Following removal of the signal peptide, the 40 kDa pro-protein is secreted *via* the ExPortal [37]. Maturation of the zymogen to mature 28 kDa cysteine protease can be accomplished by autocatalytic processing, trypsin, subtilisin or the mature SpeB protease, and involves several intermediate forms [41]. Autocatalytic activation of proSpeB occurs under reducing conditions by intra- and intermolecular mechanisms and involves a series of sequential cleavages within the 12 kDa N-terminal pro-sequence domain [29]. The pro-sequence domain also acts as an intramolecular chaperone and directs the folding of the mature protease [42]. In the present study, the secretion of proSpeB was not affected by the lack of HtrA. However, the conversion of the zymogen to the active form was significantly impaired in HSC5Δ*htrA*. We hypothesize that disruption of the *htrA* gene in *S. pyogenes* may delay or interrupt the accessory factor-mediated activation of SpeB. Several accessory proteins, including the peptidyl–prolyl isomerase RopA (also known as trigger factor) [19, 30] and LuxS [43], have been implicated in the secretion and maturation of proSpeB to the enzymatically active form. Recently, Ma *et al.* [44] demonstrated that *prsA*, a gene located immediately downstream of *speB*, is cotranscribed with *speB* and encodes for a peptidyl–prolyl isomerase, PrsA, which is required for the full conversion of proSpeB to active SpeB protease. The peptidyl–prolyl *cis–trans* isomerase (PPIase) protein family, of which PrsA and trigger factor (RopA) are members, assist the folding of nascent polypeptides *in vivo* by catalysing the *cis*/*trans* isomerization of peptidyl–prolyl bonds, which is often a rate-limiting step in protein folding [45]. In this investigation, trigger factor was unable to convert proSpeB to mature SpeB *in vitro* in the presence or absence of HtrA. To account for this observation, we suggest that the accessory proteins necessary for proSpeB maturation may act synergistically. Furthermore, these proteins may require the correct physiochemical environment of the cell wall in which to function.

The study by Lyon and Caparon [12] suggested that HtrA is essential for the maturation of proSpeB to the active protease. In the present report, we demonstrate that loss of HtrA does not impede the secretion of proSpeB and that SpeB maturation does occur without HtrA, albeit at a reduced rate. Differences in the culture conditions and the sampling of supernatant at different growth phases may account for this disparity. Furthermore, we show that HtrA does not directly convert proSpeB to active SpeB *in vitro*. Purified recombinant HtrA cleaved the model substrate β-casein, which is largely unstructured in solution [46], but was unable to directly process proSpeB.

In summary, we demonstrate that the HtrA of *S. pyogenes* does not directly process proSpeB *in vitro*. The absence of HtrA delays the conversion of proSpeB to the active form, which influences the proteolytic degradation of surface-displayed and secreted GAS virulence factors. The localization of HtrA to the GAS ExPortal [13], and the data described in this work, suggests that HtrA may influence the secretion of accessory factors required for SpeB maturation. Elucidation of the function of intrinsic ExPortal proteins, such as HtrA, may further our understanding of protein transport pathways in Gram-positive bacteria.

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