Original Article

INFECTIOUS Microbes & Diseases

OPEN

The Group A *Streptococcus* Interleukin-8 Protease SpyCEP Promotes Bacterial Intracellular Survival by Evasion of Autophagy

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Abstract

Autophagy serves an innate immune function in defending the host against invading bacteria, including group A *Streptococcus* (GAS). Autophagy is regulated by numerous host proteins, including the endogenous negative regulator calpain, a cytosolic protease. Globally disseminated serotype M1T1 GAS strains associated with high invasive disease potential express numerous virulence factors and resist autophagic clearance. Upon in vitro infection of human epithelial cell lines with representative wild-type GAS M1T1 strain 5448 (M1.5448), we observed increased calpain activation linked to a specific GAS virulence factor, the interleukin-8 protease SpyCEP. Calpain activation inhibited autophagy and decreased capture of cytosolic GAS in autophagosomes. In contrast, the serotype M6 GAS strain JRS4 (M6.JRS4), which is highly susceptible to host autophagy-mediated killing, expresses low levels of SpyCEP and does not activate calpain. Overexpression of SpyCEP in M6.JRS4 stimulated calpain activation, inhibited autophagy, and significantly decreased bacterial capture in autophagosomes. These paired loss- and gain-of-function studies reveal a novel role for the bacterial protease SpyCEP in enabling GAS M1 evasion of autophagy and host innate immune clearance.

Keywords: group A *Streptococcus*; SpyCEP; virulence factor; epithelial cells; intracellular survival; autophagy; xenophagy; calpain

Introduction

Streptococcus pyogenes, also known as group A *Streptococcus* (GAS), is a Gram-positive human pathogen associated with a spectrum of mild to life-threatening diseases, ranging from pharyngitis and impetigo, to invasive sepsis and necrotizing fasciitis; over 500,000 deaths annually worldwide are linked to GAS infections and its complications.[1,2] Although GAS is often found extracellularly in the host, it is well recognized that the pathogen can reside intracellularly, circumvent bacterial killing and replicate in human macrophages,[3–6] neutrophils[7,8] and epithelial cells.[9] Invasion of host cells and intracellular survival of GAS, despite innate host defenses, may facilitate GAS penetration into deeper tissues or the bloodstream and development of severe clinical infections.

A resurgence of invasive GAS infections over the past 40 years is largely correlated with a globally disseminated clone of the M1T1 serotype.[1,10] During invasion of the host, serotype M1T1 GAS can undergo spontaneous mutations in the CovRS 2-component system that lead to the strong transcriptional upregulation of multiple virulence factors, including streptolysin O, streptococcal inhibitor of complement, streptokinase A, and the interleukin-8 (IL-8) protease, SpyCEP (also known as ScpC), each of which promotes GAS evasion of the host innate immune mechanisms. Furthermore, mutations in the CovRS locus can suppress expression of streptococcal pyrogenic exotoxin B (SpeB), a broad-spectrum secreted cysteine protease that cleaves several GAS virulence factors.[11,12]

SpyCEP is a serine protease that cleaves and inactivates ELR motif–positive chemokines such as CXCL8/IL-8, CXCL1/Gro-α and CXCL6/GCP2.[13–16] In addition to this enzymatic activity, SpyCEP promotes adhesion to and internalization within host endothelial cells[17] and epithelial cells.[18] These properties help GAS resist host neutrophil-mediated bacterial clearance, and SpyCEP has a key role in bacterial dissemination and its expression level is associated with human invasive disease severity.[19]

One important intrinsic innate immune mechanism allowing epithelial cells to capture and clear invading intracellular bacteria is autophagy,[20] sometimes termed xenophagy in reference to clearance of a foreign infectious agent. Historically, autophagy was first recognized in eukaryotic cells as a highly conserved, intracellular system that targets, captures and degrades cytosolic components, including protein aggregates and damaged organelles.[21] More recently, studies identified that autophagy serves an innate immune defense function by targeting and clearing cytosolic bacteria such

Editor: Stijn van der Veen

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Author contributions: RB, TS, SK, GSC, VN, MR, and SU designed the study; GG, FA, and ASZ performed protein expression and purification; RB, GG, and SU performed in vitro assays; RB, FA, ASZ, VN, MR, and SU wrote the manuscript; and all authors reviewed and approved the final manuscript.

Funding: This work was supported in part by Marie Skłodowska-Curie actions Intra-European Fellowships (MC-IEF-329520), The Naito Foundation, The Takeda Science Foundation, and the US National Institutes of Health (R01-AI145325). The funders had no role in the study design, data collection or analysis, decision to publish, or preparation of the manuscript.

Conflicts of interest: The authors reported no conflicts of interest.

Infectious Microbes & Diseases (2022) 4:3

Received: 3 February 2022 / Accepted: 14 June 2022 /First published online: 26 July 2022

http://dx.doi.org/10.1097/IM9.0000000000000098

as GAS, *Mycobacterium tuberculosis*, *Salmonella typhimurium* and *Streptococcus pneumoniae*.[22–25] Some pathogenic bacteria circumvent their own autophagic elimination by means of virulence factors that (i) inhibit their recognition by the autophagy machinery, (ii) interfere mechanistically with autophagy functions, or (iii) alter autophagy regulation.[26–29] Indeed, GAS evades recognition by the autophagy machinery in part by expressing cysteine protease SpeB that degrades host ubiquitylated adapter proteins, such as p62, nuclear dot protein 52 kDa (NDP52) and neighbor of BRCA1 (NBR1), which mark bacteria for destruction.[30]

Autophagy is regulated by numerous host factors, including positive and negative regulatory proteins. Positive regulators include autophagy related 5 (ATG5) and Beclin1, whereas negative regulators include calpains, a group of cytosolic calcium-dependent cysteine proteases.[31–33] Calpains regulate a wide range of cellular processes by cleaving host proteins and are postulated to inhibit autophagy by degrading the positive regulators ATG5 and Beclin1, as well as cleaving and activating Gsα, a heterotrimeric G protein subunit, although the precise mechanisms have yet to be elucidated.[32–36] Modulation of host calpain during bacterial infection has been noted in previous reports. *Shigella flexneri* switches off host sumoylation by activation of calcium/calpain during epithelial cell infection.[37] Activation of calpain in macrophages infected with *Listeria monocytogenes* is linked to its pore-forming toxin listeriolysin O,[38] whereas GAS cytolytic toxin streptolysin S influences calpain intracellular localization, resulting in degradation of cell junction proteins and allowing GAS translocation through epithelial cell barriers.[39,40] Group B *Streptococcus* activates calpain in macrophages, leading to disruption of actin and microtubule cytoskeleton and increased apoptosis.[39,41] Here, we demonstrate that GAS SpyCEP activates calpain to decrease targeting by the autophagy pathway, thereby enhancing GAS intracellular survival.

Results

SpyCEP mediates M1.5448 autophagy evasion

Intracellular survival in host cells is an important pathogenic step in the development of invasive GAS infection. We set out to investigate key virulence factors that promote GAS intracellular survival. After screening several isogenic gene knockout strains constructed for GAS strain M1.5448, we observed that a Δ*cepA* knockout mutant survived significantly less well than the wild-type (WT) strain in cultured HEp-2/HeLa epithelial cells (Figure 1A). As one key intracellular killing mechanism active in HEp-2/HeLa epithelial cells is autophagy, we used fluorescence microscopy to compare the efficiency by which the different mutant strains of M1.5448 were captured within autophagosomes. HEp-2/HeLa cells stably transfected with microtubule-associated protein light chain 3 (LC3)–green fluorescent protein (GFP) were infected with GAS, fixed and evaluated for bacteria that colocalized with the LC3 autophagosome marker. By this assay, the percentage of intracellular M1.5448 captured in autophagosomes was significantly lower (5%–10%) compared with strain M6.JRS4, known to be highly susceptible to autophagic killing (25%–30%). In contrast, the M1.5448 Δ*cepA* mutant associated significantly more with LC3-GFP (~20%) than the M1.5448 parent strain (Figure 1B). For gain of function, we cloned and overexpressed the encoding *cepA* gene into M6.JRS4, and the resulting M6.JRS4 (M6.JRS4 p*cepA*) transformant showed significantly reduced association with LC3-GFP (10%–15%) (Figure 1C). SpyCEP expression levels in the various strains were assessed by Western immunoblot (Figure 1D). M6.JRS4 had a low SpyCEP expression level compared with WT M1.5448, whereas SpyCEP overexpression was confirmed in M6.JRS4 p*cepA*. Higher expression of SpyCEP in M1.5448 and M6.JRS4 p*cepA* correlated with decreased LC3

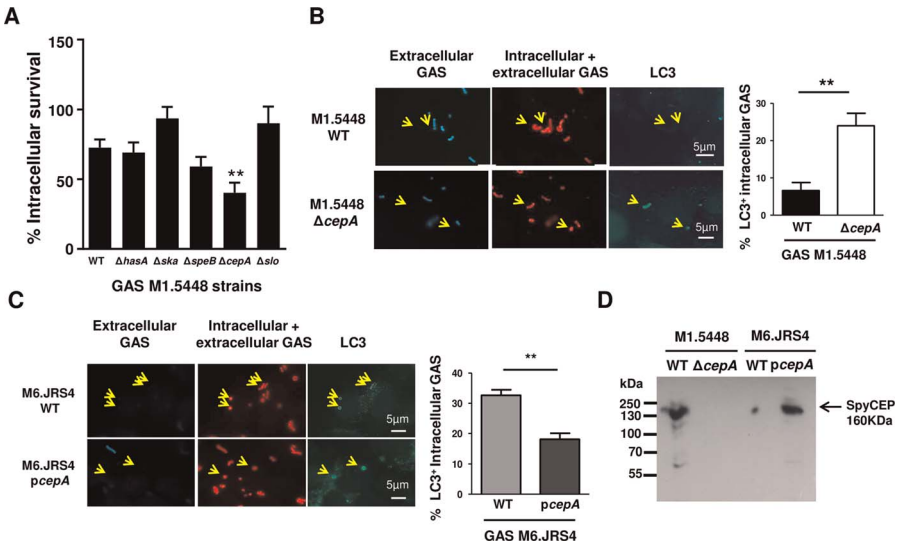

Figure 1. SpyCEP mediates evasion from autophagy in the GAS serotype M1 clone. HEp-2/HeLa epithelial cells were infected with different gene knockout strains of GAS at MOI = 10 bacteria/cell. A: Survival of bacteria within the endothelial cells was measured after 2 hours of HEp-2/HeLa cell infection and 2 and 24 hours of treatment with gentamicin, which kills extracellular bacteria. Intracellular bacterial CFUs at 4 hours after inoculation was defined as baseline CFU. % survival was intracellular CFU at 24 hours compared with baseline CFU. B: Representative immunofluorescence microscopy pictures of intracellular M1.5448 and M1.5448 Δ*cepA*. C: Representative immunofluorescence microscopy pictures of intracellular M6.JRS4 and M6.JRS4 p*cepA*. Colocalization with LC3 was calculated by counting at least 100 GAS per condition from at least 7 random pictures recorded per sample. Graph shown in (B) and (C) is a total from 3 independent experiments. Scale bar, 5 μm. D: Western blot analysis of SpyCEP expression in lysed GAS strains. *$P < 0.05$, **$P < 0.01$; one-way ANOVA with Bonferroni multiple comparisons test (A), unpaired *t* test (B and C). CFU, colony forming units; GAS, group A *Streptococcus*; LC3, microtubule-associated protein light chain 3; MOI, multiplicity of infection; WT, wild-type.

targeting, whereas loss of SpyCEP expression in the M1.5448 Δ*cepA* mutant increased its trapping in autophagosomes.

M1.5448 SpyCEP activates calpain in HEp-2/HeLa epithelial cells

Calpain is a protease and major endogenous autophagy inhibitor through cleavage and activation of a G protein subunit Gsα,[32] and by degrading autophagy-activating proteins such as ATG5 and Beclin1.[34,35] To determine if GAS SpyCEP impacted calpain activation, its activity was measured in HEp-2/HeLa epithelial cells upon infection with GAS strains. Calpain activity significantly increased after infection with M1.5448 (Figure 2A), and a role of SpyCEP in calpain activation was confirmed as the M1.5448 Δ*cepA* mutant showed reduced activation. The M1.5448 Δ*cepA* mutant was complemented with a plasmid expressing either WT SpyCEP (p*cepA*) or with a previously constructed inactive form of the peptidase (p*cepA**).[18] HEp-2/HeLa cell infection with M1.5448 Δ*cepA* (M1.5448 p*cepA*) significantly restored activation of intracellular calpain, whereas complementation with the dead enzyme version M1.5448Δ*cepA* (M1.5448 p*cepA**) did not, indicating that SpyCEP enzymatic activity is required for calpain activation (Figure 2A). The low SpyCEP-expressing GAS strain M6.JRS4 did not stimulate calpain activation, whereas the same strain M6.JRS4 p*cepA*, overexpressing SpyCEP, boosted calpain activity significantly (Figure 2B).

To test the direct effect of the SpyCEP peptidase in HEp-2/HeLa cells independently of other bacterial components, recombinant SpyCEP was directly transfected into HEp-2/HeLa cells. Introduction of SpyCEP by transfection stimulated calpain activation (Figure 2C), but intracellular expression of inactive SpyCEP* did not. Simple exposure of HEp-2/HeLa cells to recombinant SpyCEP (Ext rSpyCEP) without transfection did not lead to calpain activation (Figure 2C). The levels of calpain 1 and calpain 2 proteins expressed in HEp-2/HeLa cells infected with GAS were measured by Western blot. No changes in calpain protein levels were observed after WT M1.5448 GAS infection or infections with other mutant strains of GAS (Figure 2D). This result indicates that the change in calpain activation was due to increased enzymatic activity and not increased protein levels.

Calpain activity depends largely on calcium (Ca^{2+}) levels; thus, we quantified the intracellular Ca^{2+} in the differentially infected cells. We observed no differences in HEp-2/HeLa cells' intracellular Ca^{2+} levels when comparing M1.5448 WT to the isogenic M1.5448 Δ*cepA* mutant (Figure 2E). SpyCEP is a potent IL-8 protease, and we probed whether IL-8 degradation might mediate calpain activation. However, IL-8-neutralizing antibodies did not influence the observed changes in calpain activation (Figure 2F). Thus, the boost in calpain activity after GAS infection does not seem to be dependent upon differential Ca^{2+} changes nor SpyCEP cleavage and inactivation of IL-8.

Calpain activation by SpyCEP leads to autophagy inhibition

To assess whether SpyCEP-mediated calpain activation facilitates GAS inactivation and evasion of autophagy, we infected LC3-GFP–transfected HEp-2/HeLa cells with GAS, in the presence or absence of a calpain inhibitor calpeptin, and measured activation of autophagy by counting the number of autophagosomal puncta. Autophagy inhibition by WT M1.5448 or M6.JRS4 p*cepA*, which have higher SpyCEP expression, was reversed by the addition of calpeptin (Figure 3A and 3B). These results were further confirmed by Western blot analysis for LC3 conversion (LC3-I to LC3-II), which indicates autophagosome formation. WT M1.5448 GAS LC3 conversion levels were comparable to uninfected cells, whereas isogenic M1.5448 Δ*cepA* had increased LC3 conversion, indicating reduced autophagy evasion (Figure 3C).

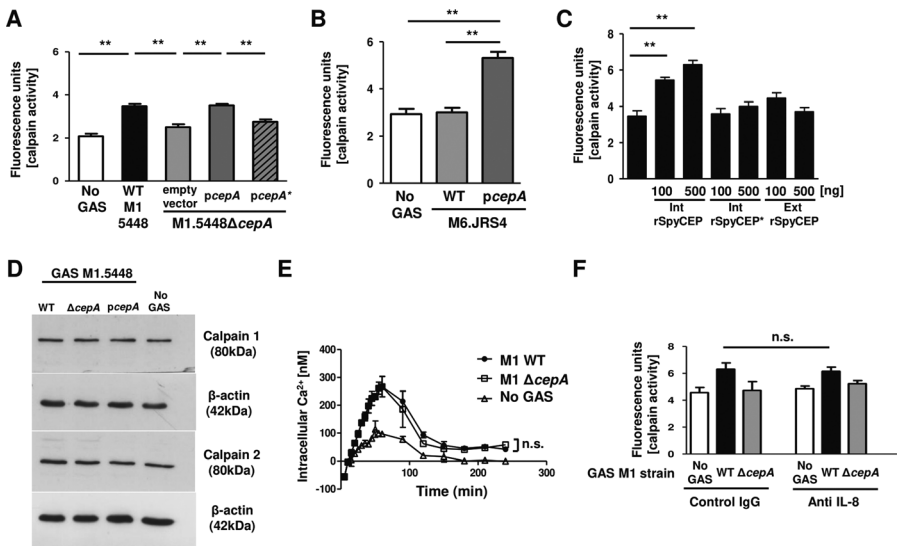

Figure 2. GAS M1 SpyCEP activates calpain in HEp-2/HeLa epithelial cells. A and B: Calpain activity in lysed HEp-2/HeLa epithelial cells infected with GAS strains with different SpyCEP expression profiles. C: Calpain activity in HEp-2/HeLa cells transfected with (Int) recombinant SpyCEP and inactive SpyCEP* and in the presence of extracellular (Ext) recombinant SpyCEP. D: Western blot on GAS-infected HEp-2/HeLa cell lysates. E: Western blot pictures are representative of results from at least 3 independent experiments. Intracellular calcium concentration in GAS-infected HEp-2/HeLa cells was measured using Fura-2. F: HEp-2/HeLa epithelial cells were either treated with 10 μg/mL anti–IL-8 neutralizing antibody or the isotype control 2 hours before infection by GAS strains. Calpain activity in lysed HEp-2/HeLa cells was measured 2 hours after infection. $^{**}P < 0.01$. n.s., not significant; one-way ANOVA with Bonferroni multiple comparisons test (A, B, E and F), two-way ANOVA with Bonferroni multiple comparisons test (C). GAS, group A *Streptococcus*; WT, wild-type.

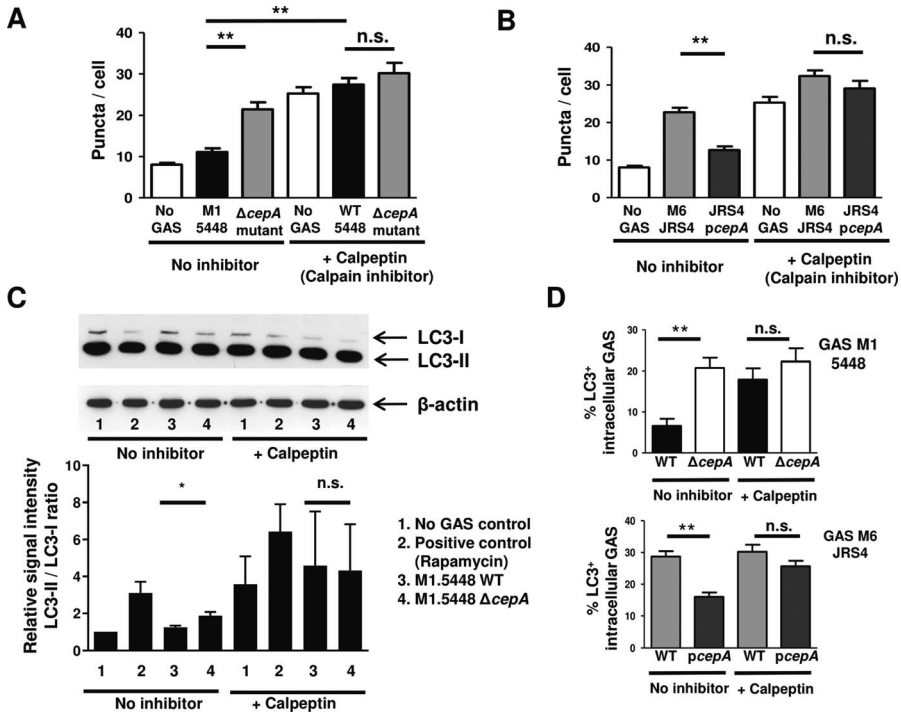

Figure 3. Calpain activation by SpyCEP leads to autophagy inhibition. A and B: Hep-2/HeLa epithelial cells stably transfected with LC3-GFP were treated or not with 50 μM calpeptin, a calpain inhibitor, for 24 hours before infection. Four hours after infection with GAS strains, activation of autophagy was analyzed by counting LC3-GFP puncta per cell on immunofluorescence microscopy pictures. C: Four hours after infection with GAS strains, activation of autophagy was analyzed by LC3 Western blot. The picture of the Western blot is a representative of 3 independent experiments. The bar graph showing signal intensity of the Western blot derived from the average of 3 experiments. D: Percentage of intracellular GAS in association with autophagosomes (colocalized with LC3-GFP) was counted and calculated. $^{*}P < 0.05$, $^{**}P < 0.01$. n.s., not significant; two-way ANOVA with Bonferroni multiple comparisons test (A and B), one-way ANOVA with Bonferroni multiple comparisons test (C), unpaired *t* test (D). GAS, group A *Streptococcus*; GFP, green fluorescent protein; LC3, microtubule-associated protein light chain 3; WT, wild-type.

Inhibition of calpain activation with calpeptin increased the association of intracellular WT M1.5448 and M6.JRS4 p*cepA* with LC3-GFP, erasing the prior differences between strains (Figure 3D). These results indicate that SpyCEP expression induces calpain activity, which helps GAS to evade autophagosomal targeting.

SpyCEP is essential for GAS evasion of host intracellular killing by autophagy

Intracellular M1.5448 successfully resides and replicates in the host cell cytoplasm, whereas the GAS strain M6.JRS4 is efficiently captured and controlled by autophagosomes.[30] To prove whether SpyCEP can promote GAS escape from autophagosomes, we examined the susceptibility of intracellular M1.5448 and its isogenic M1.5448 Δ*cepA* mutant to penicillin, an antibiotic that enters into the cytosol, but not into compartments of the endocytic pathway, such as autophagosomes.[42] First, infected HEp-2/HeLa cells were treated with gentamicin to kill all extracellularly located bacteria, and intracellular replication and survival of the M1.5448, M1.5448 Δ*cepA*, M6.JRS4 and M6.JRS4 p*cepA* strains were assessed. We found that SpyCEP-expressing strains M1.5448 and M6.JRS4 p*cepA* replicated intracellularly, whereas the M1.5448 Δ*cepA* mutant and M6.JRS4 did not (Figure 4A). HEp-2/HeLa cell viability after these infection conditions did not differ statistically between WT (79% ± 2%) and Δ*cepA* (78% ± 3%) strains.

These data indicate that SpyCEP promotes GAS intracellular persistence and replication. When infected HEp-2/HeLa cells were treated with a combination of gentamicin and penicillin to kill extracellular bacteria, WT M1.5448 was cleared more efficiently than the M1.5448 Δ*cepA* mutant (~70% vs 30% within 10 hours from start of treatment; Figure 4B), suggesting M1.5448 localization to the cytosol rather than within autophagosomes. Thus, SpyCEP enables GAS to escape capture in autophagosomes and to reside and multiply in the cytosol.

Addition of 3-methyladenine (3-MA), a compound that blocks autophagosome formation via the inhibition of type III phosphatidylinositol 3-kinases (PI-3 K),[3] 2 hours before bacterial infection allowed the M1.5448 Δ*cepA* mutant to replicate intracellularly as efficiently as the M1.5448 WT strain, and the M6.JRS4 WT strain to survive as efficiently as the M6.JRS4 p*cepA* strain (Figure 4C and 4D). These results again support a capacity of autophagy to defend against intracellular bacterial replication, whereas SpyCEP allows GAS to subvert autophagy-mediated killing.

Discussion

Autophagy can be deployed as an innate immune mechanism to target, capture and kill cytosolic and vacuole-associated bacteria, a process sometimes designated xenophagy. Several important bacterial pathogens have developed mechanisms to circumvent this host defense. For example, *S. typhimurium* inhibits the signaling cascade that initiates autophagy,[43] *S. flexneri* masks itself with host proteins to evade recognition by the autophagy machinery,[28] and *Staphylococcus aureus* inhibits fusion with lysosomes to survive and replicate within autophagosomes.[44] Barnett et al.[30] reported

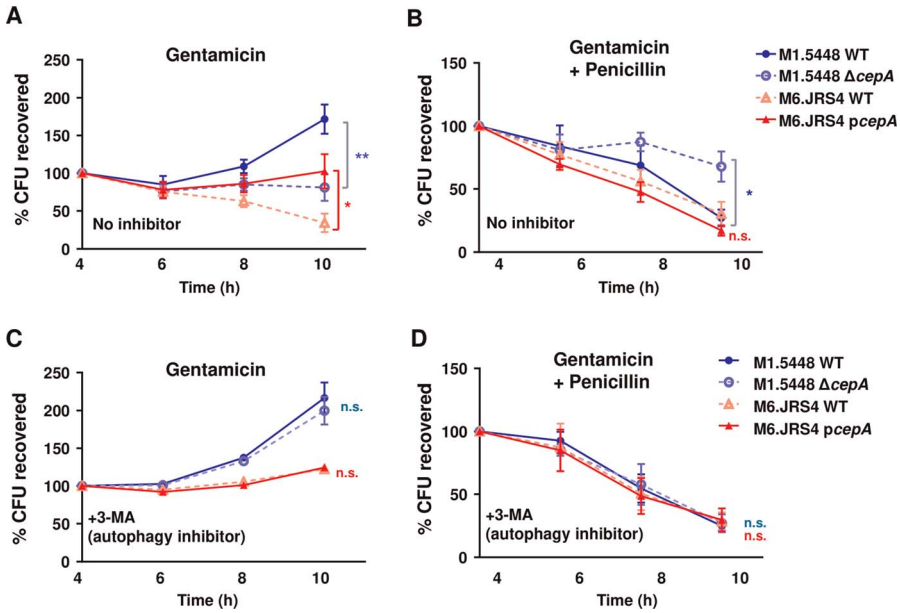

Figure 4. SpyCEP is essential for GAS evasion of host intracellular killing by autophagy. Intracellular growth and survival of GAS strains in HEp-2/HeLa cells was analyzed, defining 4 hours postinfection as baseline for comparison at later time points. A: Intracellular survival of GAS strains with extracellular killing (gentamicin). B: Intracellular survival of GAS strains with extracellular and cytoplasmic killing (gentamicin + penicillin). C: Intracellular survival of GAS strains with and without autophagy inhibition (3-MA, 5 mM) gentamicin + 3-MA. D: Intracellular survival of GAS strains with and without gentamicin + penicillin + 3-MA. *$P < 0.05$, **$P < 0.01$. n.s., not significant; one-way ANOVA with Bonferroni multiple comparisons test, M1.5448 compared with M1.5448 Δ*cepA* and M6.JRS4 compared with M6.JRS4 p*cepA*. 3-MA, 3-methyladenine; CFU, colony forming units; GAS, group A *Streptococcus*; WT, wild-type.

that GAS strain M1.5448 is resistant to autophagy-mediated killing, in contrast to strain M6.JRS4, which was used extensively in early studies of autophagy-mediated innate immunity against GAS. One mechanism by which GAS M1 decreases recognition by the autophagy machinery is expression of the cysteine protease SpeB. This protease degrades host ubiquitylated adapter proteins, such as p62, NDP52 and NBR1, which normally bind to both ubiquitin and the ubiquitin-like protein LC3, linking ubiquitin-tagged bacteria to the autophagic machinery.[45] In this study, we identify an additional mechanism by which GAS M1 escapes autophagy. GAS protease SpyCEP activates the host protease calpain, a known endogenous autophagy inhibitor, to block autophagy and avoids capture in autophagosomes.

We provide several lines of evidence in support of this conclusion. (i) Targeted mutagenesis of *spyCEP* decreased GAS activation of host calpain, increased activation of autophagy, and increased GAS association with the LC3 autophagosome marker; (ii) inhibition of calpain with calpeptin increased activation of autophagy and increased association of intracellular GAS with LC3; (iii) purified SpyCEP activated calpain and inhibited autophagy; and (iv) overexpression of SpyCEP in the autophagy-susceptible M6.JRS4 strain boosted host calpain activation and resistance to autophagy. We thus conclude that SpyCEP, previously recognized as an IL-8 peptidase and mediator of adhesion to host endothelial cells,[18] has an additional role in GAS evasion of autophagic clearance.

Prior studies by our laboratory and others have shown that both SpeB and SpyCEP may be essential to GAS survival within the host, depending on the context of infection. GAS causes a wide array of superficial and invasive diseases and expresses different virulence factors during superficial versus invasive infections. For example, M1 GAS SpyCEP is highly upregulated during invasive infection, whereas SpeB can be downregulated.[46] The complementary functions of the 2 proteases may assist GAS in evading autophagy during both superficial mucosal and later invasive stages of diseases.

One of the main factors that activate calpain in host cells is intracellular calcium. We did not see differences in intracellular calcium levels of the HEp-2/HeLa cells infected with M1.5448 WT and M1.5448 Δ*cepA*. Epithelial cells secrete IL-8 upon bacterial entry,[47] and because SpyCEP is an enzyme that cleaves and inactivates IL-8, we hypothesized that IL-8 might be a trigger for host calpain activation. Inhibition of IL-8 activity using a neutralizing antibody did not change calpain activation measured in HEp-2/HeLa cells infected with M1.5448 WT and M1.5448 Δ*cepA*. Thus, the exact mechanism by which SpyCEP activates host calpain remains unknown. We have shown that the enzymatic activity of SpyCEP is required to activate calpain, suggesting that proteolytic degradation of host proteins may be involved. However, the host factor(s) with which SpyCEP directly interacts has yet to be identified. Calpain also influences other host factors and processes that could impact GAS invasion.

Group B *Streptococcus* (*Streptococcus agalactiae*) induces calpain activation in macrophages, resulting in cleavage of cytoskeleton proteins and macrophage membrane permeability disruption.[41] One GAS virulence factor, the pore-forming toxin streptolysin S, facilitates GAS invasion into deeper tissues via degradation of epithelial intercellular junctions, which results from recruitment of calpain to the plasma membrane.[40] Activation of calpain by SpyCEP might have roles in GAS pathogenesis beyond autophagy inhibition, a potential topic for future studies.

The M6.JRS4 strain is a laboratory-adapted strain previously described to have low or deficient expression of many virulence factors.[30,48] Here, we show that this strain also has a very low expression of SpyCEP (Figure 1D). Lack of this major virulence factor certainly increases its susceptibility to autophagy-mediated killing,

consistent with the impaired virulence of this strain in mice.[49] By activating host calpain and inhibiting autophagy, the globally disseminated GAS M1T1 WT strain evades autophagy-mediated killing in epithelial cells. This may represent a critical mechanism for the pathogen in establishing invasive infections.

Materials and methods

Bacterial strains

All GAS strains were grown in Todd-Hewitt medium (BD Bacto, Franklin Lakes, New Jersey) with 0.2% yeast extract (BD Bacto) (THY) at 37 °C. These include GAS strains M6.JRS4,[50] M1.5448, mutant M1.5448 Δ*cepA*,[13] complemented strain M1.5448 Δ*cepA* (M1.5448 p*cepA*) with SpyCEP expression restored by plasmid complementation using the expression plasmid p*cepA*, M1.5448 Δ*cepA* (M1.5448 p*cepA**) expressing an inactive form of SpyCEP, SpyCEP*[18] and M6.JRS4 p*cepA* constructed by introducing plasmid p*cepA*[13] into M6.JRS4 by electroporation.

Cell culture and transfections

Cell lines were cultured at 37 °C in 5% CO_2. HEp-2 cells are epithelial cell lines derived from HeLa cell contamination (ATCC); thus, we call these cells Hep-2/HeLa cells throughout.[20–53] Hep-2/HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS; PAA Laboratories GmbH). GFP-LC3–expressing plasmid DNA was introduced into HEp-2/HeLa cells by electroporation using the Amaxa Nucleofector kit (Lonza AG, Basel, Switzerland) following the manufacturer's instruction. Transfection of recombinant SpyCEP and SpyCEP* into HEp-2/HeLa cells was performed using PULSin protein delivery reagent (Polyplus transfection, Illkilch, France) following the manufacturer's instruction.

Infection of cultured cells

GAS strains grown to optical density (OD) at 600 nm (OD_{600}) of 0.4 were used to inoculate confluent HEp-2/HeLa cells at a multiplicity of infection (MOI) of 10. After 2 hours, gentamicin, which kills extracellular bacteria, was added to the wells with or without penicillin, which kills both extracellular and free cytosolic bacteria, depending on the experiments performed.

Calpain activity measurement

Calpain activity was measured using a commercial assay kit (Abcam, Cambridge, Massachusetts) following the manufacturer's instruction. Briefly, after 4 hours of infection by GAS strains, HEp-2/HeLa cell lysate was incubated with the substrate (Ac-LLY-AFC) and reaction buffer for 1 hour at 37 °C in the dark. Calpain cleavage of the substrate was measured by fluorescence at a wavelength of 505 nm after excitation at 400 nm.

IL-8 neutralization

HEp-2/HeLa cells were treated with 10 μg/mL anti–IL-8 neutralizing antibody (Abcam) or isotype control (Abcam) 2 hours before infection. The calpain activity assay was performed as described above.

Intracellular free calcium measurement

Host intracellular free calcium was measured using the fluorescence Ca^{2+} indicator dye Fura-2 following the protocol previously described.[28] In brief, HEp-2/HeLa cell monolayers were cultured to semiconfluence in 96-well flat bottom plates. Cells were incubated in phenol red–free DMEM + 4 mM probenecid (Sigma) + 5 mM Fura-2, AM (Abcam) + 10% pluronic F-127 (Sigma, St. Louis, Missouri) for 30 minutes at 37 °C. After incubation, cells were washed 3 times with culture media before infection with GAS strains at an MOI of 100. The infected monolayer was monitored at 37 °C in a Varioscan Flash fluorescent plate reader (Thermo Scientific, Waltham, Massachusetts) every 5 minutes for 240 minutes with excitation and emission wavelengths of 380 and 510 nm, respectively. Intracellular free Ca^{2+} was calculated by the equation $[Ca^{2+}]_i = K_d[(R - R_{min})/(R_{max} - R)]$. The maximal fluorescence ratio (R_{max}) was determined by adding 0.1% Triton X-100, and the minimal fluorescence ratio (R_{min}) was determined by subsequent addition of 5 mM BAPTA.

Antibodies

Primary antibodies used were rabbit polyclonal antisera raised against whole GAS bacteria (anti-GAS), rabbit anti-LC3B (Novus Biologicals Littleton, Colorado), monoclonal rabbit anti-LC3B (D11) XP (Cell Signaling, #3868; antibody dilution 1:1000), rabbit anti-actin (Sigma-Aldrich, A5060; antibody dilution 1:1000), rabbit anti-SpyCEP, rabbit anti–calpain 1 large subunit and rabbit anti–calpain 2 large subunit (Cell Signaling, #2556 and #2539, respectively; antibody dilution 1:1000). Secondary antibodies used for immunofluorescence microscopy were Alexa Fluor–conjugated goat anti-rabbit antibodies (Invitrogen, A-11046, A-21244; dilution 1:300). Secondary antibodies used for Western blots were horseradish peroxidase (HRP)–conjugated polyclonal goat anti-rabbit antibody (Dako, P0448; antibody dilution 1:3000).

Recombinant SpyCEP purification

The expression vector for SpyCEP in *Escherichia coli* was a gift from Dr Susanne R. Talay. Expression of the *SpyCEP* gene was induced for 4 hours at 30 °C and 120 rpm using isopropylthio-β-galactoside (IPTG: 1mM) after the strain had reached an OD of 0.5 to 0.6. Bacterial cells were then centrifuged using a Sorvall centrifuge at 3000 to 8000 rpm at 4 °C, washed twice with cold phosphate-buffered saline (PBS), treated with lysis buffer and protease inhibitor, then lysed using a FrenchPress. The cell lysate was centrifuged at 13,000 rpm for 15 minutes at 4 °C, the supernatant loaded onto an Ni-proteino column (Macherey-Nagel), and the protein was purified following the manufacturer's protocol. Recombinant SpyCEP was dialyzed in PBS overnight.

Western blot

HEp-2 cells were grown in 12-well plates to 70% to 80% confluency. After GAS infection, samples were lysed by the addition of 150 μL of 1× sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris, 2% wt/vol SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% wt/vol bromphenol blue), heated for 5 minutes at 95 °C, run on SDS-PAGE, and electro-transferred to nitrocellulose. Blocking was performed for 1 hour at room temperature in blocking buffer [1× Tris-buffered saline (TBS; Sigma), 0.05% Tween-20, 5% wt/vol nonfat dry milk]. After washing in TBS-T (1× TBS, 0.05% Tween-20), the membrane was incubated overnight at 4 °C with primary antibodies in 1× TBS-T, 5% bovine serum albumin (dilutions mentioned above). After washing in 1× TBS-T, the membranes were incubated with HRP-conjugated secondary antibodies for 1 hour at room temperature. Signal detection of the washed membranes was performed with the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and exposure to x-ray films.

SpyCEP Western blot

GAS strains grown to an OD_{600} of 0.4 were washed 3 times with PBS and were resuspended in 100 µL of DMEM at a concentration of 1×10^9 colony forming units (CFU)/mL. Mutanolysin (Sigma) was added at a concentration of 2500 U/mL, and the samples were incubated at 37 °C for 1 hour. After incubation, 5× SDS sample buffer was added to the samples followed by heating for 5 minutes at 95 °C. For each sample, 15 µL was run per well on an SDS-PAGE following the protocol described above.

Immunofluorescence microscopy

HEp-2/HeLa cells were cultured on glass coverslips and infected with GAS strains as described above. For positive controls, cells were treated with 50 nM rapamycin for 18 hours. At 4 hours post-infection, coverslips were washed 4 times with Hanks' balanced salt solution and fixed in 1% paraformaldehyde (PFA). After several washes, coverslips were mounted onto glass slides with ProLong Gold anti-fade reagent with DAPI (Molecular Probes). The activation of autophagy was analyzed by counting LC3-GFP$^+$ puncta. To analyze intracellular GAS in autophagosomes, fixed cells were blocked in PBS containing 10% FBS. Extracellular bacteria were stained with anti-GAS rabbit antiserum followed by Alexa Fluor 350–conjugated goat anti-rabbit antibody (1:300; Invitrogen). Coverslips were again blocked and permeabilized in 10% FBS + 1% PFA + 0.3% Triton X-100 in PBS for 60 minutes and stained with GAS rabbit antisera followed by Alexa Fluor 647–conjugated goat anti-rabbit antibodies (1:300; Invitrogen) in PBS + 10% FBS + 0.3% Triton X-100. Coverslips were then mounted onto glass slides using ProLong Gold anti-fade reagent (Molecular Probes). Stained samples were examined using Zeiss Axio Imager A2 with a Zeiss AxioCam MRm camera and ZEN 2011 software at ×1000 magnification with oil immersion. At least 7 random pictures (average 15 cultured cells/picture) were recorded per sample.

GAS intracellular growth assay

HEp-2/HeLa cells were infected with GAS strains as described above. For inhibition of autophagy, the cells were treated with 5 mM 3-MA 2 hours before bacterial infection. After 2 hours, cells were washed once with 10% FBS + DMEM and then incubated in 10% FBS + DMEM with 100 mg/mL gentamicin to kill extracellular bacteria. After 2 hours, the cells were washed 3 times with 10% FBS + DMEM. Cells from 3 wells were lysed and plated in serial dilutions to determine intracellular GAS CFUs used as a baseline. All the other wells were incubated with media containing gentamicin alone or gentamicin + 100 U/mL penicillin G. After different incubation times, cells were washed 3 times with PBS, treated with 100 mL of trypsin, and lysed by the addition of 0.025% Triton X-100. Percent CFU recovered after 24 hours was calculated considering 2 hours as 100%. Cell lysates were serially diluted and plated on blood agar plates for bacterial enumeration.

Statistics

For statistical analysis, the mean ± standard error of the mean for 3 independent experiments is shown in figures unless otherwise stated. We analyzed numeric data for statistical significance using Student unpaired *t* test or analysis of variance (ANOVA) with Prism software (GraphPad). *P* values of less than 0.05 were considered as statistically significant. Significance are presented as $^{**}P < 0.01$ or $^{*}P < 0.05$.

Acknowledgments

We thank Stephanie Peter for additional experimental assistance and Shannon Weiman for critical reading. SpyCEP antibody was provided by Dr Susanne R. Talay.

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How to cite this article: Bergmann R, Gulotta G, Andreoni F, Sumitomo T, Kawabata S, Zinkernagel AS, Chhatwal GS, Nizet V, Rohde M, Uchiyama S. The group A *Streptococcus* interleukin-8 protease spycep promotes bacterial intracellular survival by evasion of autophagy. *Infect Microb Dis* 2022;4(3):116–123. doi: 10.1097/IM9.0000000000000098