The Globally Disseminated M1T1 Clone of Group A Streptococcus Evades Autophagy for Intracellular Replication

Timothy C. Barnett,1,2* David Liebl,3 Lisa M. Seymour,1,2 Christine M. Gillen,1,2 Jin Yan Lim,1,2 Christopher N. LaRock,4 Mark R. Davies,1,2,5 Benjamin L. Schulz,1,2 Victor Nizet,4 Rohan D. Teasdale,1,3 and Mark J. Walker1,2*

1Australian Infectious Diseases Research Centre
2School of Chemistry and Molecular Biosciences
3Institute for Molecular Bioscience
The University of Queensland, Queensland, St Lucia, QLD 4072, Australia
4Department of Pediatrics and Skaggs School of Pharmacy & Pharmaceutical Sciences, University of California, San Diego, La Jolla, CA 92093, USA
5Wellcome Trust Sanger Institute, Hinxton, Cambridge CB10 1SA, UK
*Correspondence: mark.walker@uq.edu.au
http://dx.doi.org/10.1016/j.chom.2013.11.003

SUMMARY

Autophagy is reported to be an important innate immune defense against the intracellular bacterial pathogen Group A Streptococcus (GAS). However, the GAS strains examined to date belong to serotypes infrequently associated with human disease. We find that the globally disseminated serotype M1T1 clone of GAS can evade autophagy and replicate efficiently in the cytosol of infected cells. Cytosolic M1T1 GAS (strain 5448), but not M6 GAS (strain JRS4), avoids ubiquitylation and recognition by the host autophagy marker LC3 and ubiquitin-LC3 adaptor proteins NDP52, p62, and NBR1. Expression of SpeB, a streptococcal cysteine protease, is critical for this process, as an isogenic M1T1ΔspeB mutant is targeted to autophagy and attenuated for intracellular replication. SpeB degrades p62, NDP52, and NBR1 in vitro and within the host cell cytosol. These results uncover a proteolytic mechanism utilized by GAS to escape the host autophagy pathway that may underpin the success of the M1T1 clone.

INTRODUCTION

Autophagy is a highly conserved cellular process that targets cytosolic components, including protein aggregates, damaged organelles, and intracellular bacteria, for lysosomal degradation, thus playing important roles in homeostasis and innate immunity (Deretic, 2010). Autophagy is an important cytosolic innate immune defense against bacterial infections (Huang and Brumell, 2009), and successful intracellular bacterial pathogens avoid autophagy by replicating in membrane-bound vacuoles or by camouflaging their surface with host- or bacterial-derived proteins (Dortet et al., 2011; Ogawa et al., 2005; Yoshikawa et al., 2009). Intracellular bacteria can be targeted to autophagy by a number of adaptor proteins that recognize polyubiquitylated bacteria in the cytosol or damaged bacteria-containing vacuoles (Kirkin et al., 2009; Thurston et al., 2009, 2012). These adaptor proteins, which include p62 (SQSTM1), NDP52 (CALCOCO2), NBR1, and optineurin, direct cargo to nascent LC3-positive phagophores and ultimately to degradation by the lysosomal pathway (Chong et al., 2012; Thurston et al., 2009; Wild et al., 2011; Zheng et al., 2009).

Group A Streptococcus (GAS) is an obligate human pathogen and the fourth most common bacterial cause of human mortality (Carapetis et al., 2005). The GAS disease burden ranges from superficial infections (pharyngitis, impetigo), to life-threatening invasive conditions (toxic shock, necrotizing fasciitis), to postinfectious immune disorders (rheumatic fever, glomerulonephritis) (Cole et al., 2011). A number of GAS strains are efficiently internalized into epithelial cells where they can be targeted to autophagy and cleared; however, these strains belong to serotypes M6 (Joubert et al., 2009; Nakagawa et al., 2004; Sakurai et al., 2010), M49 (Joubert et al., 2009), and M89 (Thurston et al., 2009), which are not representative of the prevalent serotypes associated with contemporary human disease epidemiology (Cole et al., 2011; Steer et al., 2009). Here, we show that the globally disseminated serotype M1T1 clone of GAS can replicate efficiently in the cytosol of infected cells through a process that involves proteolysis of the host proteins that target intracellular bacteria to autophagy.

RESULTS

M1T1 Strain 5448 Replicates within Epithelial Cells and Avoids Autophagy

While GAS has served as a model organism to unravel the complex molecular events that lead to antibacterial autophagy, the strains examined belong to serotypes infrequently associated with human disease. We therefore compared the intracellular survival of one such laboratory-adapted M6 strain (strain JRS4, hereafter M6RS4) (Nakagawa et al., 2004) with a recent clinical isolate of the globally disseminated serotype M1T1 clone (strain 5448, hereafter M1T15448) that has been the single leading cause of both pharyngitis and severe invasive GAS infections during the...
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last three decades. Intracellular viability of GAS following entry into human HEP-2 epithelial cells was monitored over time by measuring colony-forming units (cfu) (Figure 1A). Consistent with prior studies, the viability of the M6<sup>RS4</sup> strain decreased over time, as only 47% of cfu present at 4 hr postinfection remained at 8 hr postinfection. In contrast, recoverable cfu of the M1T1<sup>5448</sup> strain tripled from 4 to 8 hr postinfection, revealing a capacity of this clinically important strain to not only survive, but also replicate, within epithelial cells.

To determine whether M1T1<sup>5448</sup> intracellular replication reflected resistance to autophagy, we performed immunofluorescence microscopy to quantitate intracellular M1T1<sup>5448</sup> or M6<sup>RS4</sup> GAS that colocalized with the autophagy marker GFP-LC3 (Figures 1B and 1C). M6<sup>RS4</sup> GAS were efficiently targeted to autophagy, with 48.1% ± 9.2% of intracellular M6<sup>RS4</sup> found in LC3-positive vacuoles at 6 hr postinfection. In contrast, only low numbers of M1T1<sup>5448</sup> GAS were transiently associated with GFP-LC3 at 2 hr postinfection, and by 6 hr postinfection, the vast majority (96.4% ± 0.7%) were negative for GFP-LC3, demonstrating that the virulent human M1T1 isolate avoids targeting to autophagy to replicate within human epithelial cells.

**M1T1<sup>5448</sup> GAS Replicate in the Cytosol of Epithelial Cells**

Autophagy primarily targets bacteria in the cytosol or in damaged membrane compartments, and bacterial pathogens such as *Salmonella* Typhimurium avoid autophagy by replicating within modified vacuoles (Birmingham et al., 2006). We therefore explored whether M1T1<sup>5448</sup> avoids autophagy by replicating within an intact vacuole. To directly visualize intracellular M1T1<sup>5448</sup> and M6<sup>RS4</sup> GAS, we performed transmission electron microscopy on GAS-infected HEP-2 cells at 6 hr postinfection (Figures 2A and 2B). The M1T1<sup>5448</sup> strain was abundantly present in the cytosol of infected cells, whereas the M6<sup>RS4</sup> strain was contained within a membrane-bound compartment. To confirm that M1T1<sup>5448</sup> was not associated with endosomal membranes, we performed immunofluorescence microscopy to quantitate the association of M1T1<sup>5448</sup> with markers of early (EEA1) and late (Lamp1) endosomes (Figure 2C and Figure S1, available online). While transiently associated with endosomes at 2 hr postinfection, only 2.3% ± 0.7% of intracellular M1T1<sup>5448</sup> were EEA1 or Lamp1 associated at 6 hr postinfection, suggesting that M1T1<sup>5448</sup> escapes the endosomal pathway to replicate.

We additionally examined the susceptibility of intracellular M1T1<sup>5448</sup> to penicillin. Penicillin is an antibiotic that rapidly enters the cytosol, but not components of the endocytic pathway (Renard et al., 1987), a property that has been used to select for *Listeria monocytogenes* mutants that fail to escape the endocytic pathway (Camilli et al., 1989). Penicillin treatment abolished the intracellular replication of WT M1T1<sup>5448</sup> with viability diminishing over the course of the experiment (Figure 2D). In contrast, penicillin treatment had minimal effect on the intracellular viability of M6<sup>RS4</sup> (Figure 2E). Taken together, these results suggest that the intracellular replication of M1T1<sup>5448</sup> likely correlates to their successful entry into the cytosol of infected cells.

**Intracellular M1T1<sup>5448</sup> GAS Avoids Ubiquitylation and the Ubiquitin-LC3 Adaptor Proteins NDP52, p62, and NBR1**

With few exceptions, ubiquitylation is a critical step in selective autophagy (Kirkin et al., 2009). LC3 is targeted to ubiquitylated bacteria via ubiquitin-LC3 adaptor proteins, which include p62, NDP52, NBR1, and optineurin (Chong et al., 2012; Deretic, 2010; Mostowy et al., 2011; Thurston et al., 2009; Wild et al., 2011). To determine whether M1T1<sup>5448</sup> escapes autophagy by avoiding these pathways, we performed immunofluorescence microscopy on M1T1<sup>5448</sup> and M6<sup>RS4</sup> infected cells to determine their respective colocalization with ubiquitylated proteins.
(Figure 3A), NDP52 (Figure 3B), p62 (Figure 3C), and NBR1 (Figure 3D). Paralleling previous observations (Thurston et al., 2009), M6JRS4 GAS were found associated with NDP52, with the proportion of GFP-LC3-positive M6JRS4 GAS associated with NDP52 gradually increasing from 13.7% ± 3.0% at 2 hr post-infection to 33.3% ± 4.3% at 8 hr postinfection. However, the majority of GFP-LC3-positive M6JRS4 GAS were found associated with p62 (82.5% ± 3.7% to 96.3% ± 3.7%) and NBR1 (92.2% ± 4.0% to 98.4% ± 1.6%) at all time points examined (Table S1). In contrast, intracellular M1T15448 GAS bacteria were not found in association with ubiquitylated proteins, NDP52, p62, or NBR1 (Figure 3), suggesting that intracellular M1T15448 actively evades the autophagy pathways in epithelial cells.

SpeB Cysteine Protease Is Required for Efficient Intracellular Replication of M1T15448 GAS

The M6JRS4 strain examined by Nakagawa et al. (2004) was previously shown to be defective in expression of the extracellular cysteine protease SpeB (Lyon et al., 2001) (Figure S2A), and is avirulent in a murine model of GAS infection (Figure S2B). Given that SpeB is a secreted and surface-associated protease (Hytonen et al., 2001) whose expression varies among GAS strains, we hypothesized that this virulence determinant may play a role in avoidance of the ubiquitylation system. Thus, we examined the ability of WT M1T15448 and an isogenic ΔspeB mutant to replicate within epithelial cells. Comparison of the genome sequences of M1T15448 and M1T15448 ΔspeB revealed that these strains differ by only two SNPs (Figure S3). Compared to the WT parent strain, the M1T15448 ΔspeB mutant was attenuated for intracellular replication (Figure 4A), but not for replication in THY broth in vitro (Figure S3A). Furthermore, immunofluorescence microscopy revealed that, in comparison to wild-type (WT) M1T15448, targeting of the M1T15448 ΔspeB mutant to autophagy was much more efficient (Figures 4B and 4C), with 25.3% ± 3.9% intracellular M1T15448 ΔspeB associated with GFP-LC3 at 6 hr postinfection.

To further investigate the role of SpeB in resistance to autophagy, we engineered a M6JRS4 strain that expresses SpeB. In contrast to M6JRS4, M6JRS4 + pSpeB replicated efficiently within HEp-2 cells (Figures 4D) and was only rarely associated with GFP-LC3 (Figure S2C). Similar results were also obtained with the SpeB-expressing strains M6JMGAS10394 (Banks et al., 2004) and M12HKU16 (Tse et al., 2012), which replicated efficiently, compared to the naturally occurring SpeB-negative M4NS244 strain (McKay et al., 2004), which failed to replicate (Figure S2D).

SpeB Degrades Ubiquitin-LC3 Adaptor Proteins NDP52, p62, and NBR1

To determine whether SpeB conferred resistance to autophagy by its broad-spectrum cysteine protease activity, we purified SpeB from M1T15448 (Figure S2E) and examined its ability to...
degrade components of the host ubiquitylation system. Purified SpeB efficiently degraded NDP52, p62, and NBR1 (Figure 4E), as well as ubiquitylated proteins (Figure S2F) from HEp-2 epithelial cell extracts. Similar results were obtained with bacterial culture supernatants from WT M1T15448, but not the isogenic ΔspeB strain (Figure S2G). To confirm that these effects were specific to SpeB, we performed parallel experiments in the presence of the cysteine protease inhibitor E64 (Cole et al., 2007) (Figures 4E, S2F, and S2G). In a manner comparable to the mock-treated samples, the E64-treated, purified SpeB was deficient in the proteolytic activities described above.

We propose the ability of SpeB to act as a defense against host ubiquitylation components relies on its proteolytic activity within the environment of the host cytosol. To confirm that SpeB was enzymatically active in the host cytosol, we transfected HEp-2 cells with plasmids encoding either codon-optimized SpeB or a catalytically inactive C192S derivative and compared their effect on NDP52, p62, and NBR1 (Figures 4F and S2H–S2J). The plasmid encoding WT SpeB significantly reduced the number of NDP52, p62, and NBR1 puncta within transfected cells when compared to the catalytically inactive C192S mutant, demonstrating that WT SpeB is enzymatically active within the host cell cytosol. Taken together, these results demonstrate that GAS SpeB protease is necessary and sufficient to degrade ubiquitylation components within the host cytosol that normally serve to direct the bacterium to autophagy.

**DISCUSSION**

Autophagy primarily targets bacteria in the cytosol or in damaged membrane vacuoles. Some intracellular bacterial pathogens, such as *Salmonella Typhimurium* (Birmingham et al., 2006) and *Staphylococcus aureus* (Schnaith et al., 2007), avoid autophagy by replicating within modified endocytic or autophagic compartments. Other bacterial pathogens, such as *Shigella flexneri* and *Listeria monocytogenes*, escape the endocytic pathway and replicate in the cytosol of infected cells, avoiding ubiquitylation by camouflaging their surface with bacterial or host-derived proteins (Dortet et al., 2011; Ogawa et al., 2005; Yoshikawa et al., 2009). Here we provide several lines of evidence that GAS employ a proteolytic mechanism to evade autophagy and replicate in the cytosol of infected cells: (1) genetic deletion of the gene encoding SpeB significantly reduced the intracellular replication of M1T15448; (2) genetic deletion of the gene encoding SpeB from the M1T15448 strain significantly increased the frequency of the recruitment of LC3 to the surface of the intracellular bacteria; (3) expression of SpeB by M6JRS4 promoted intracellular replication and abolished recruitment of LC3 to the bacterial surface; (4) purified SpeB degrades the ubiquitin-LC3 adaptor proteins p62, NDP52, and NBR1; (5) ectopic expression of codon-optimized SpeB in HEp-2 cells reduced the amounts of p62, NDP52, and NBR1; and (6) WT SpeB-expressing strains replicate efficiently in epithelial cells while naturally occurring SpeB-defective strains fail to replicate. Therefore, we propose that production of a bacterial protease that degrades the host proteins responsible for targeting bacteria to autophagy, in addition to proteolytic degradation of ubiquitylated bacterial surface proteins, constitutes a previously unrecognized mechanism employed by a bacterial pathogen to evade autophagy.

GAS is a highly successful pathogen of its human host, causing a wide array of superficial and invasive diseases. The transition from superficial to invasive disease involves a genetic
switch that abolishes SpeB expression while concomitantly increasing expression of numerous virulence factors required for growth in deeper tissues (Cole et al., 2011). As such, there is a strong correlation between SpeB expression and superficial disease (Cole et al., 2011; Ikebe et al., 2010). SpeB is a broad-spectrum cysteine protease required for virulence (Cole et al., 2006) that has been shown to degrade a number of immunologically important human proteins, including immunoglobulins, chemokines, proinflammatory cytokines, and cathelicidin, and to proteolytically modify GAS surface proteins such as M protein, chemokines, proinflammatory cytokines, and cathelicidin, which is important for host colonization (Johansson et al., 2008; Nelson et al., 2011). Additionally, SpeB has been shown to bind to a variety of host proteins, including laminin (Hyttinen et al., 2001) and integrins (Stockbauer et al., 1999), which may have undescribed influences on host cell differentiation and signaling. Together, these properties suggest that SpeB expression is required by GAS for multiple aspects of epithelial colonization, which includes resistance to autophagy and other innate defenses.

In addition to SpeB expression, there is also a strong correlation of certain GAS serotypes with superficial disease in developed countries; M1T1 and M12 strains are the most prevalent in published epidemiological studies (Shulman et al., 2009). In contrast, M6 (Joubert et al., 2009; Nakagawa et al., 2004; Sakurai et al., 2010), M49 (Joubert et al., 2009), and M89 (Thurston et al., 2009), which have been used in prior GAS autophagy studies, are much less frequently associated with human disease. It is likely that M1T1 and other prevalent serotypes possess other genetic traits that enhance their success at colonizing epithelial surfaces (Maamary et al., 2012). In agreement with this is our observation that the M1T15448 speB mutant associated with GFP-LC3, at 4 hr postinfection. Arrows indicate intracellular M1T15448 ΔspeB mutant associated with GFP-LC3. Bar = 5 μm.

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In conclusion, we report here a previously unrecognized strategy employed by an intracellular bacterial pathogen to manipulate the host autophagy pathway. By production of a protease that degrades host proteins that target bacteria to autophagy, a strain of the globally disseminated M1T1 GAS serotype can evade autophagy and replicate efficiently in the cytosol of infected epithelial cells. We propose that SpeB is required by GAS strains to establish successful colonization of epithelial tissues, thereby allowing such strains to escape an important component of host innate defense.

**EXPERIMENTAL PROCEDURES**

**Bacterial Growth**

GAS strains M6^Rfr^ (Nakagawa et al., 2004), M1T1^S448^ (Kansal et al., 2003), M1T1^S448ΔspeB^ (Kansal et al., 2003), M6^AGAS10354^ (Banks et al., 2004), M12^KU16^ (Tse et al., 2012), and M4^N524^ (McKay et al., 2004) were grown in Todd-Hewitt medium with 0.2% yeast extract (THY) at 37°C. Strain M6^Rfr^ ΔpSpeB was constructed by introducing plasmid pSpeB (Korotkova et al., 2012) into M6^Rfr^ by electroporation.

**Cell Culture and Transfections**

Cell lines were cultured at 37°C in 5% CO2. HEp-2 cells were originally obtained from ATCC and grown in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, Gibco). Plasmid DNA was introduced into HEp-2 cells using Attractene reagent (QIAGEN).

**GAS Intracellular Growth Assay**

For GAS infection, early stationary-phase bacteria (OD600 = 1.0–1.2) were harvested and washed once with Dulbecco’s phosphate buffered saline (DPBS [pH 7.4]). The bacteria were diluted into 1% FBS/DMEM and added to confluent cell culture monolayers at a multiplicity of infection equal to 0.8 (eight bacteria for every ten cells). After 2 hr, cells were washed once with 10% FBS/DMEM and then incubated for 10% FBS/DMEM with 100 µg/ml gentamicin to kill extracellular bacteria. Where indicated, 100 µM penicillin G was also included. After an appropriate incubation time, infected cells were washed twice with PBS, treated with 200 µl trypsin, and lysed by the addition of 0.025% Triton X-100. Cell lysates were serially diluted and plated on THY/C14 medium and dialyzed extensively against PBS.

**SpeB Activity Assay**

HEp-2 cell lysates were prepared by lysing confluent monolayers in T75 flasks (~8 × 10^6 cells) in 150 µl DPBS containing 1% Triton X-100, 0.1% SDS, and EDTA-free complete protease inhibitor (Roche). Lysates were passed ten times through a 27G needle and insoluble material removed by centrifuging at 14,000 × g for 15 min. The resulting supernatant was dialyzed extensively against DPBS before use.

SpeB was purified from stationary-phase M1T1^S448^ culture supernatants using a modification of the protocol described by Berge and Björck (1996). Briefly, SpeB was precipitated from a 500 ml 24 hr culture supernatant of M1T1^S448^ with 80% w/v ammonium sulfate. The suspended precipitate was dialyzed against 5 mM MES (pH 6.0) and purified by affinity chromatography on SP-Sephadex column eluted with a 5–250 mM MES (pH 6.0) stepwise gradient and dialyzed extensively against PBS.

**Statistics**

For statistical analysis, the mean ±SEM for three independent experiments is shown in figures unless otherwise stated, and p values were calculated using a one-tailed Student’s t test. A p value of less than 0.05 was determined to be statistically significant.
ACCESSION NUMBERS
The Illumina genome sequence data of M1T15448 and M1T15448 SpeB were deposited into the ENA (http://www.ebi.ac.uk/ena/) under the accession numbers ERSS1322 and ERSS1323, respectively.

SUPPLEMENTAL INFORMATION
Supplemental Information includes three figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2013.11.003.

ACKNOWLEDGMENTS
Microscopy was carried out at the Australian Cancer Research Foundation (ACRF) Cancer Biology Imaging Facility at the Institute for Molecular Bioscience. Electron microscopy was carried out at The Centre for Microscopy and Microanalysis (the Queensland Node of the Australian Microscopy and Microanalysis Research Facility). We thank Ericka Anderson for additional experimental assistance. This work was supported by funding from the National Health and Medical Research Council (NHMRC) of Australia (1041294, 631386, 635250, 565526, 1041929, and 606788) and the National Institutes of Health (NIH) of the United States of America (AI077780, AI057153, and AR052728).

Received: July 9, 2013
Revised: October 10, 2013
Accepted: October 28, 2013
Published: December 11, 2013

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