Mycobacterium tuberculosis Alters the Metalloprotease Activity of the COP9 Signalosome

Lia Danelishvili,a,b Lmar Babrak,a,b Sasha J. Rose,a,b Jamie Everman,a,b Luiz E. Bermudeza,b Department of Biomedical Sciences, College of Veterinary Medicine,a and Department of Microbiology, College of Science,b Oregon State University, Corvallis, Oregon, USA

L.B. and S.J.R. contributed equally to this work.

ABSTRACT Inhibition of apoptotic death of macrophages by Mycobacterium tuberculosis represents an important mechanism of virulence that results in pathogen survival both in vitro and in vivo. To identify M. tuberculosis virulence determinants involved in the modulation of apoptosis, we previously screened a transposon bank of mutants in human macrophages, and an M. tuberculosis clone with a nonfunctional Rv3354 gene was identified as incompetent to suppress apoptosis. Here, we show that the Rv3354 gene encodes a protein kinase that is secreted within mononuclear phagocytic cells and is required for M. tuberculosis virulence. The Rv3354 effector targets the metalloprotease (JAMM) domain within subunit 5 of the COP9 signalosome (CSN5), resulting in suppression of apoptosis and in the destabilization of CSN function and regulatory cullin-RING ubiquitin E3 enzymatic activity. Our observation suggests that alteration of the metalloprotease activity of CSN by Rv3354 possibly prevents the ubiquitin-dependent proteolysis of M. tuberculosis-secreted proteins.

IMPORTANCE Macrophage protein degradation is regulated by a protein complex called a signalosome. One of the signalosomes associated with activation of ubiquitin and protein labeling for degradation was found to interact with a secreted protein from M. tuberculosis, which blocks the complex and inactivates it. The interference with the ability to inactivate bacterial proteins secreted in the phagocyte cytosol may have crucial importance for bacterial survival within the phagocyte.
tiapoptotic behavior of \textit{M. tuberculosis} (15). We demonstrated that \textit{M. tuberculosis} is capable of blocking the extrinsic pathway of apoptosis by secreting the Rv3654c and Rv3655c effectors, which alter the caspases’ posttranscriptional events (15). We also identified the secreted Rv3364c protein, which inhibits caspase-1 activation and consequently host cell apoptosis (pyroptosis) through suppression of the enzymatic activity of cathepsin G (16). In the present study, we characterized the function of the Rv3354 gene and demonstrated for the first time the novel virulence mechanism of \textit{M. tuberculosis} in which the secreted Rv3354 exploits the host ubiquitylation system by altering COP9 signalosome function to limit the degradation of \textit{M. tuberculosis} effector proteins.

**RESULTS**

Characterization of the Rv3354 gene knockout mutant. The 2G2 mutant (Fig. 1A), which lacks the ability to inhibit macrophage apoptosis, was identified from a transposon bank of \textit{M. tuberculosis} mutants (15). Sequencing analysis revealed that transposon insertion at the 105-amino-acid (aa) site disrupted proper translation of Rv3354 (Fig. 1B). Bioinformatic analysis of the Rv3354 protein revealed domains of DUFF732 (unknown function) and PKc_MEK1 (the catalytic domain of the dual-specificity protein kinase mitogen-activated protein kinase/extracellular signal-regulated kinase 1 [MAPK/ERK1]). Using the sequenced-based prediction for secreted proteins and SignalP 4.1, the presence of a 32-aa signal peptide and export via the Sec system were predicted for Rv3354. Complementation of the 2G2 mutant (Rv3354\textsuperscript{+}/H11001) restored the antiapoptotic phenotype (Fig. 1C). We next examined 2G2 for survival in THP-1 cells. \textit{In vitro} studies revealed no difference between growth of \textit{M. tuberculosis} H37Rv wild type (WT) and growth of 2G2 in liquid culture medium (Fig. 1D); however, the Rv3354 knockout clone showed a significant decrease in growth within macrophages (Fig. 1E). The viability was fully recovered by complementing 2G2 with the functional Rv3354 gene. Table 1 shows the comparison in apoptosis and intracellular bacterial growth for the \textit{M. tuberculosis}-infected primary macrophage cell line. The results confirmed that apoptosis and bacterial growth happen in monocyte-derived macrophages (MDM) in a similar way as in THP-1 cells.

The Rv3354 protein is secreted within human macrophages. We recently established a fluorescent screening tool for mycobacterium-secreted proteins by using a TEM-1

---

**FIG 1** Inactivation of the \textit{M. tuberculosis} Rv3354 gene. (A) Genetic organization of the Rv3354 gene in \textit{M. tuberculosis} strain H37Rv. (B) The signal peptide, predicted domains, and Tn5367 insertion site in the Rv3354 protein. (C) Apoptosis was analyzed in THP-1 cells infected with WT, 2G2, and 2G2 (Rv3354\textsuperscript{+}) in a cell death detection ELISAPLUS assay (Roche). Results represent means ± standard errors of the means of three independent experiments. **, \(P < 0.01\); *, \(P < 0.05\), for the significance of differences between 2G2 and WT. (D) \textit{In vitro} growth of WT, 2G2, and 2G2 (Rv3354\textsuperscript{+}) in aerated 7H9 medium. (E) Infection and impaired growth of 2G2 in THP-1 cells. WT, 2G2 and 2G2 (Rv3354\textsuperscript{+}) were used at an MOI of 10:1. The significance of differences between 2G2 and WT survival, recorded with bacterial CFU at days 3 and 5 of infection, was \(P < 0.01\) (**).
β-lactamase reporter enzyme (Bla) and a mammalian fluorescence resonance energy transfer (FRET)-based probe which can be used to monitor Bla enzyme activity in cultured cells. The positive-control bla+ vector with a full-length β-lactamase gene, the negative-control bla-deficient vector lacking a signal sequence of the bla gene important for β-lactamase enzyme secretion, and the experimental vector containing an Rv3354:bla-deficient fusion were transformed into the M. tuberculosis-lactam-sensitive PM638 clone. Bla protein secretion was monitored in THP-1 cells via hydrolysis of the FRET-based β-lactamase substrate CCF2-AM by using fluorescence microscopy (Fig. 2A) as well as a cytofluorometer (Fig. 2B). The infection rate of M. tuberculosis-infected THP-1 cells was found to be similar in all groups (Fig. 2C).

**TABLE 1** Comparison of the level of apoptosis and intracellular M. tuberculosis growth in infected human MDM

| Infection† | % Apoptosis‡ | CFU/ml of lysate |
|------------|--------------|------------------|
|            | 1 day | 3 days | 5 days | 1 day | 3 days | 5 days |
| WT         | 8 ± 3 | 15 ± 4 | 38 ± 2 | 5.9 × 10⁵ | 2.7 × 10⁵ | 3.4 × 10⁵ |
| 2G2        | 11 ± 3 | 35 ± 1 | 55 ± 4 | 5.7 × 10⁵ | 8.9 × 10⁵ | 1.6 × 10⁶ |
| 2G2 (Rv3354+) | 9 ± 2 | 18 ± 3 | 37 ± 5 | 5.4 × 10⁵ | 2.3 × 10⁵ | 3.6 × 10⁵ |
| No infection | 3 ± 1 | 5 ± 3 | 14 ± 2 |

†Approximately 5 × 10⁵ macrophages were infected with M. tuberculosis at an MOI of 10.
‡The results represent the means ± SD of two assays and were determined in an ELISA.

**FIG 2** Rv3354 protein secretion within THP-1 cells. (A) THP-1 monolayers in a 96-well plate were loaded with CCF2-AM and then infected with M. tuberculosis expressing bla-deficient, bla+, or Rv3354c:bla-deficient vector at an MOI of 10:1. While uncleaved substrate emits green, β-lactamase-catalyzed substrate fluoresces blue. Bar, 10 μm. (B) CCF2-AM hydrolysis readings from THP-1 cells infected with M. tuberculosis bla-deficient, bla+, or Rv3354c:bla-deficient vector also confirmed the robust changes between control and experimental groups, which were recorded with a plate reader. The secretion of β-lactamase was captured at 8 h postinfection. (C) The percentage of M. tuberculosis-infected macrophages as a function of the number of ingested M. tuberculosis with bla-deficient, bla+, or Rv3354c:bla-deficient vector after 1 h of incubation.

**Effectors Rv3354 interacts with the metalloprotease (JAMM) domain of CSN5.** To identify the host protein(s) targeted by the effector Rv3354, we performed a yeast two-hybrid screen with a human universal cDNA library and with the M. tuberculosis target protein as the bait. Out of 2 × 10⁷ transformants screened, 21 clones grew in the absence of tryptophan (-Trp), leucine (-Leu), histidine (-His), and adenine (-Ade). Eighteen clones were further eliminated through screening on 125 ng/ml aureobasidin plates in the presence of 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-Gal) and absence of Ade, His, Leu, and Trp. Three interacting positive clones were sequenced and, two out-of-frame clones were further eliminated. A 48- to 261-aa coding sequence of CSN5 was identified as an interacting partner with Rv3354, and at 68- to 154-aa JAMM domain sequence was detected (Fig. 3A). Further studies using full and reverse constructs of the bait and prey targets confirmed a positive interaction between JAMM and Rv3354 (Fig. 3B, panels a and b). When we mutated JAMM (mJAMM) in the amino acid sequences essential for its metalloprotease activity, neither bait nor pray constructs showed a positive interaction, suggesting that JAMM is a specific target for the M. tuberculosis effector.

The effector Rv3354 colocalizes and binds to CSN5 in THP-1 cells. To demonstrate direct binding of Rv3354 to JAMM, we...
In vitro interaction between Rv3354 and CSN5 proteins. (A) Predicted JAMM domain and mutated sites in the CSN5 protein. The amino acids Glu^{77} and Arg^{106}, responsible for metalloprotease activity of CSN5, were replaced with Ala and Gly by using a site-directed QuikChange mutagenesis kit according to the manufacturer’s protocol (Stratagene). (B) The yeast two-hybrid interaction of Rv3354 with the host target protein. (a) The full-length CSN5 and, mainly, its JAMM domain showed a positive interaction with Rv3354. (b) Reverse screening of the Rv3354 interaction with JAMM or mJAMM. (C) Subcellular localization of Rv3354 and the JAMM motif of CSN5 in THP-1 cells. dtTomato:Rv3354 and ZsGreen:JAMM viral particles were coexpressed transiently in THP-1 cells for 24 h. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Bar, 10 μm. (D) Coimmunoprecipitation of Rv3354 and CSN5 proteins. The recombinant 6×HN:CSN5 or mutated version of a protein was incubated with purified M. tuberculosis proteins overexpressing Rv3354 with Flag tag. The bound proteins were captured with His columns and subjected to IB with 6×HN and Flag antibodies.
analyzed bacterial and host protein colocalization within macrophages by using a lentiviral transfection system (Fig. 3C) and performed a coimmunoprecipitation assay (Fig. 3D). Following infection of THP-1 cells with lentiviral particles of the fusions tdTomato:Rv3354 and ZsGreen:JAMM or ZsGreen:mJAMM, samples were analyzed with fluorescence microscopy. ZsGreen:JAMM-expressing cells showed granular fluorescence in the cytosol and mainly colocalized with dtTomato:Rv3354-expressing cells, as shown in the merged image in Fig. 3C. Conversely, mutated ZsGreen:mJAMM-expressing cells showed diffuse and uniform cytoplasmic staining with Rv3354 protein. The control interaction between tdTomato and ZsGreen:JAMM displayed a dispersed location as well.

We also performed coimmunoprecipitation of Flag-tagged Rv3354 (Flag:Rv3354) with 6×HN:CSN5 (6×HN:CSN5). Recombinant 6×HN:CSN5 with or without mutations in JAMM was expressed in the pET system. The Flag:Rv3354 fusion was overexpressed in pMV261 and transformed into M. tuberculosis. Bacteria were lysed at the mid-log growth phase, and the cleared protein fraction was incubated with the recombinant 6×HN:CSN5  overnight at 4°C. The CSN5 protein was purified using His columns, and bound proteins were visualized by Western blotting. The mutated recombinant 6×HN:mCSN5 protein was used as a negative control in these experiments. Purification of CSN5 led to copurification of Rv3354 protein (Fig. 3D). No interaction was detected between mutated CSN5 and Rv3354, explaining the lack of physical binding between host and bacterial proteins.

Rv3354 binding to JAMM alters the function of Cullin-based E3 ubiquitin ligases. To examine if the Rv3354 interaction with CSN5 leads to any modification of the host protein, we performed Western blot analysis of CSN5 on whole-cell lysates of THP-1 macrophages infected with the WT, 2G2 mutant, or a complemented clone. We did not observe any cleavage or changes in the molecular mass of CSN5 (Fig. 4A). Next, we asked whether the interaction of Rv3354 with the metalloprotease domain altered CSN5 function. JAMM directly binds to the Nedd8 protein, a key facilitator of ubiquitin-protein isopeptide ligase (E3) complex assembly, and it cleaves Nedd8 from Cul-RING E3 ligases, a process known as deneddylation (17). This process is essential for efficient recycling progression by E3 enzymes. The Western blot analysis of cullin1 (Cul1), cullin3 (Cul3), and associated Nedd8 protein was carried out in WT-, 2G2- or 2G2 (Rv3354/H11001)-infected macrophages. As shown in Fig. 4B, while immunoblotting (IB) of Cul1 and Cul3, derived from 2G2-infected THP-1 cells, revealed a nearly complete deneddylation of Cul1 and Cul3 at all time points, the wild-type and complemented 2G2 infections partially blocked the deneddylation process. The loss of Nedd8 was apparent in the positive-control group, which was cells treated with 50 μM N-ethyl-carbamidoadenosine (NECA) (Fig. 4B).

Modification of Cul3-based E3 ubiquitin ligase activity mediates caspase-8 inhibition during M. tuberculosis infection. The cul3-based E3 ligase interaction with DISC (death-inducing signaling complex) has been demonstrated to induce Cul3-mediated polyubiquitination of caspase-8, promoting its full activation and apoptosis (18). To examine whether alteration in the Cul3 deneddylation process by M. tuberculosis results in changes of caspase-8 polyubiquitination and, alternatively, its activation, we performed a caspase-8 immunoprecipitation assay with extracts of THP-1 cells infected with either WT or 2G2 followed by Western blotting with caspase-8 or ubiquitin (Ub) antibody. The results indicated that the amount of ubiquitinated caspase-8 in WT-infected cells markedly decreased at 48 h compared with 2G2 infection (Fig. 4C, panels a and b). The amount of activated p18
Kinase activity of Rv3354 protein. (A) Kinase activity of recombinant Rv3354 was assessed by fluorescently measuring ADP production as a direct result of kinase phosphotransferase activity from ATP. Results represent means ± standard errors of three independent experiments. **, P < 0.01, significance of differences between experimental Rv3354 and the reaction buffer control. (B) THP-1 cells were infected with WT, 2G2, and 2G2 (Rv3354*) for 4, 8, and 24 h, and cleared protein samples were subjected to CSN5 immunoprecipitation using agarose-conjugated primary antibody. Total CSN5 was IB with anti-phospho-serine/threonine/tyrosine antibody. (C) IB analysis of PSF protein in WT- and 2G2-infected macrophages at 4, 8, 12, 24, or 48 h postinfection.

Rv3354 demonstrates protein kinase activity. Bioinformatic analysis of the Rv3354 protein revealed a catalytic domain of the MAP/ERK1 protein on the C terminus. To determine if Rv3354 possesses any kinase activity, phosphotransferase activity was measured by ADP production. Briefly, ATP, as a kinase substrate, was added to the recombinant 6×H8:Rv3354 protein (200 μg/ml) or bacterial or host total protein extracts (650 μg/ml; positive controls). As shown in Fig. 5A, the experimental wells that contained purified Rv3354 produced significantly more ADP than the reaction buffer (negative control) alone, demonstrating that when ATP is a substrate, the Rv3354 protein possesses kinase activity.

CSN5 has a reduced level of phosphorylation in the absence of Rv3354. To evaluate the effect of Rv3354, as a potential protein kinase, on CSN5 activity, we analyzed CSN5 phosphorylation during M. tuberculosis infection. Figure 5B shows increasing amounts of CSN5 at 4 h postinfection in WT- and 2G2 (Rv3354*)-infected macrophages, while 2G2-infected cells had significantly lower CSN5 protein levels at 4 h and 8 h postinfection. Over time, the phosphorylation of CSN5 markedly decreased in H37Rv- and 2G2 (Rv3354*)-infected cells, whereas CSN5 phosphorylation levels increased in 2G2 mutant-infected cells at 24 h postinfection (Fig. 5B).

Modulation of CSN5 function most likely protects M. tuberculosis effectors from Ub degradation. The most-characterized biological process that the COP9 signalosome regulates via JAMM is the cleavage of the ubiquitin-like protein Nedd8 from Cul-containing E3 ligases. The neddylation and deneddylation of Cul-containing E3 ligase is a strategy of the bacterium to avoid ubiquitination of its effector proteins. To further examine this possibility, we infected cells with WT and 2G2 and performed IB analysis for the polyipyrimidine tract-binding protein-associated splicing factor (PSF). Previously, we demonstrated that the anti-apoptotic secreted Rv3654c protein, nonfunctional in the 31G12 clone, binds to the PSF in macrophages and cleaves the host protein. This, in turn, results in decreased levels of caspase-8 activity, which leads to inhibition of the intrinsic pathway of apoptosis (15). In the current study, we observed that the 2G2 mutant, in contrast to WT infection but similarly to infection with the Rv3654c knockout clone (31G12), failed to cleave the PSF protein (Fig. 5C). Our observation suggests the possibility that the Rv3654c effector is degraded in cells infected with 2G2 (lacking Rv3354) and, as a result, fails to cleave PSF.

DISCUSSION

Host-driven apoptotic death of macrophages during bacterial infection appears to be an essential aspect of innate immunity aimed at the elimination of niche cells on which bacteria rely for replication and survival (19). Modulation of apoptosis is one of the mechanisms whereby M. tuberculosis avoids death by macrophages (20). Less is known about the M. tuberculosis virulence factors that are involved in control of the apoptosis process, although M. tuberculosis antiapoptotic phenotypes in host cells have been well documented (11, 19, 20) and de novo protein synthesis has been suggested to control an antiapoptotic activity in both macrophages and epithelial cells (11). We identified an M. tuberculosis transposon clone with a nonfunctional Rv3354 gene as deficient in the inhibition of macrophage apoptosis and observed that Rv3354 is required for M. tuberculosis virulence and survival in macrophages. By constructing the Rv3354 signal sequence fusion with the β-lactamase reporter gene and utilizing the mammalian cell-based reporter assay, we demonstrated Rv3354 protein secretion within THP-1 phagocytic cells. Complementary to our findings, other groups have shown Rv3354 protein export in culture filtrates of M. tuberculosis H37Rv and experimentally confirmed its secretion via a phoA’ reporter system in vitro (21, 22). Furthermore, protein-protein interaction studies identified the metalloprotease (JAMM) motif within CSN5 as an interacting partner for Rv3354. CSN is implicated in diverse biological functions, including apoptosis (23). In particular, when CSN5, an essential subunit for CSN metalloprotease activity, is deleted from the COP9 complex, it leads to apoptotic cell death in vivo (23). In addition, CSN5 can stably exist independently from the complex.
As an independent apoptotic mechanism from CSN, CSN5 can enhance apoptosis via activation of transcription factor E2F1 (23). CSN5 regulates the cullin-ring ligase (CRL) families of ubiquitin E3 complexes by cleaving (via neddylation) a ubiquitin-like protein, Ned8, from the cullin ring. Many bacterial pathogens have developed diverse strategies of interference with the host ubiquitin-proteasome system and, in several cases as a consequence, alter the apoptosis process (24, 25). Evidence suggests that effector proteins such as NleG (Escherichia coli O157:H7), LubX and SidH (Legionella pneumophila), SopA and SspH2 (Salmonella), and IpaH3 (Shigella) mimic and function as E3 ubiquitin ligases in the host (24). In contrast, effectors like SseL (Salmonella), TssM (Burkholderia pseudomallei), YopJ (Yersinia pseudotuberculosis and Yersinia pestis), and YopP (Yersinia enterocolitica) exhibit deubiquitylation activities that manipulate regulatory components of the host ubiquitin system and apoptosis (25, 26). In some cases, as a part of the intracellular activation mechanism of effector proteins, for example, with ExoU (Pseudomonas), the host ubiquitin is used as a cofactor for enzymatic activation (27).

In this work, we have provided evidence that host ubiquitin is used as a cofactor for enzymatic activation (27). Of effector proteins, for example, with ExoU (Pseudomonas), the host ubiquitin is used as a cofactor for enzymatic activation (27). In some cases, as a part of the intracellular activation mechanism of effector proteins, for example, with ExoU (Pseudomonas), the host ubiquitin is used as a cofactor for enzymatic activation (27). In our work, we have provided evidence that M. tuberculosis-secreted protein Rv3354 possesses protein kinase activity, and we have demonstrated that the interaction of Rv3354 with the JAMM motif changes a phosphorylation profile of CSN5 in M. tuberculosis-infected cells. The recent work has implicated a functional relationship between the CSN and protein kinase enzymes, and this relationship determines CSN stability toward the ubiquitin system. In fact, immunoprecipitation and far-Western blotting experiments have revealed that CSN copurifies with protein kinases and serves as a docking site for the phosphorylation of various substrates (28). Analysis of CSN5-associated kinase-specific phosphorylation sites has revealed several serine and threonine residues as putative phosphate acceptors, and mutations in these sites have confirmed its importance in activation of CSN (17, 29). In addition, the treatment of cultured cells with the piceatannol, an inhibitor of CSN-associated kinases, has an effect on CSN5 function (30). A genetic inactivation of the Rv3354 gene leads to decreased activation of CSN, which is normally enhanced with the wild-type M. tuberculosis. The significant phosphorylation of CSN5 at early time points suggests that the pathogen attempts to inactivate the proteasome system by blocking the deneddylation process of enzymes that is responsible for enabling this system. Our results demonstrate that the most prominent Cul1 and Cul3 ligases of CSN5 in WT-infected cells lead to accumulation of Ned8-conjugated enzymes. In addition, it has been established that p62-dependent Cul3-mediated polyubiquitination and activation of caspase-8 mediate extrinsic apoptosis signaling (18). We found that when the Rv3354 knockout mutant failed to destabilize the Cul3 enzyme in a similar manner as the WT, it led to a significantly higher level of caspase-8 polyubiquitination. Similar to M. tuberculosis, the Vpr protein of human immunodeficiency virus type 1 (HIV-1) has been implicated in regulation of apoptosis via interaction with CSN (31). Alternatively, the cycle-inhibiting factors (Cif) of many pathogenic bacteria have been shown to directly interact with several Ned8-conjugated cullins and to inhibit ubiquitin ligase activity, altering 26S proteasome-mediated degradation (32).

Why would a pathogen target host ubiquitin-mediated protein degradation? Recent reports suggest that bacterial modulation of the host proteasome system is a mechanism to program the elimination of effector proteins when the bacterium’s function within the host cell is no longer required, therefore avoiding permanent damaging effects in the host cell (26). In fact, certain pathogens modulate this system to temporarily prevent the destruction of active effectors. This strategy also ensures safe synthesis and efficient export of secreted proteins into the host cells. For example, temporal regulation has been reported for Salmonella Rho GTPase-modulating enzymes, SopE and SptP, and for Legionella pneumophila E3 ligases LubX and SidH (27). Our hypothesis is that by modifying the signalosome function, M. tuberculosis might also enhance the activity of many other effector proteins delivered to host cells at the same time as Rv3354. In this work, by using the Rv3354 knockout mutant, we demonstrated that M. tuberculosis fails to cleave the host PSF protein in a similar fashion as the 31G12 knockout mutant of the Rv3654c gene (15). This observation suggests that when M. tuberculosis fails to impair CSN activity, secretion of the Rv3654c effector (responsible for direct modification of PSF) is inefficient, most likely due to protein degradation.

The successful reprogramming of cellular survival or death pathways by M. tuberculosis, which influences host immune responses, has been mainly justified through the ESX-1 secretion system and its ESAT-6 and CFP-10 effectors. However, recent evidence shows that blocking the secretion of these effectors does not always result in M. tuberculosis attenuation in vitro and in vivo (33). A growing literature has revealed new virulence factors that influence the intracellular survivability of M. tuberculosis. In the current study, we characterized the Rv3354 virulence effector that is required for M. tuberculosis survival in macrophages. The underlying mechanism of the Rv3354-CSN5 interaction as a cause of apoptosis remains unclear; however, an indirect mechanism cannot be excluded at this time. Based on our work, we can conclude that M. tuberculosis alters the CSN function and limits E3 ligase activity via Rv3354, and this possibly prevents the ubiquitin-mediated protein degradation of M. tuberculosis effector proteins. This finding adds another layer of complexity to M. tuberculosis virulence and provides insight into future research that will help to elucidate a novel mechanism of M. tuberculosis pathogenicity.

MATERIALS AND METHODS

Antibodies and systems. The primary antibodies against CSN5, Cul1, Cul3, caspase-8, Ub, PSF, β-actin, phosphoserine/threonine/tyrosine of human origin, and Flag and 6×H1N probes were purchased from Santa Cruz Biotechnology. All chemicals were obtained from Sigma. The GenBLAzer cell-based assay loading kit was from Life Technologies. The yeast two-hybrid Normalized Mate and Plate universal human library, the yeast transformation system, and the bait, prey, and control vectors were obtained from Clontech. The Lenti-X lentiviral expression system was also purchased from Clontech.

Cell and bacterial cultures. The THP-1 human monocyte cell line was obtained from the American Type Culture Collection (ATCC) and maintained in RPMI 1640 medium (Lonza) supplemented with heat-inactivated 10% fetal bovine serum (FBS) and 2 mM l-glutamine. To promote maturation and adherence, cells were treated with 100 ng/ml of phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich) and then seeded at 80% confluence into 75-cm² tissue culture flasks, 24-well plates, or chamber glass slides, as needed. After 24 h, cells were replenished with new medium and incubated for an additional 48 h for cell differentiation. The M. tuberculosis G2 mutant, G2 (Rv3354Δ), and wild-type H37Rv, purchased from ATCC, were grown until mid-exponential phase in Middlebrook 7H9 broth supplemented with 10% albumin-dextrose-catalase, 0.2% glycerol, and 0.05% Tween 80. Where appropriate, kanamycin was added at a concentration of 200 µg/ml. M. tuberculosis cells were homog-
enzined to remove clumps, and only dispersed inocula were used in infec-
tion experiments. The viable counts of inocula were determined by serial
dilution and plating on 7H11 agar with 10% oleic acid-albumin-dextrose-
catalase. Human MDM were obtained from the blood of volunteers by
using protocols approved by the Institutional Human Subject Commit-
tee. The purification, culture, and maturation of these cells were described
previously (34). Cells were cultured in RPMI 1640 medium supplemented
with 10% autologous serum. In all experiments, macrophages were in-
fected at a multiplicity of infection (MOI) of 10. CFU were calculated at
different times postinfection. In another set of experiments, apoptosis was
measured via a cell death detection enzyme-linked immunosorbent assay
(ELISAPLUS; Roche) or cells were cleared and analyzed by Western blot-
ting for selected host proteins.

Complementation of 2G2. The PCR-generated 390-bp coding frag-
ment of Rv3354 was cloned into the mycobacterium shuttle vector pMV261-ApII containing the apramycin resistance marker. The result-
ating vector was electroporated into 2G2. Transformants were plated on
agar plates containing 200 μg/ml of apramycin and screened for positive
clones by PCR using apramycin primers (15).

β-lactamase assay for protein secretion. A 32-aa coding sequence of
the Rv3354 gene was cloned into the mycobacterium-E. coli shuttle vector
pLGD13 downstream of the G13 promoter of Mycobacterium tuberculosis
and upstream of the bla gene lacking the first 69-bp signal sequence. The
resultant vector was transformed into the blaC knockout strain PM638 of
M. tuberculosis H37Rv. Approximately 10^7 THP-1 cells were seeded into
96-well plates or in 2-chamber slides and infected with M. tuberculosis
clones expressing the full-length bla, the bla-deficient mutant missing
the signal sequence, or the Rv3354:bla-deficient fusion. After a 1-h incu-
bation at 37°C in 5% CO2, extracellular bacteria were removed by washing
wells with Hanks’ balanced salt solution. Infected control and experimen-
tal monolayers were loaded with the cell-permeable dye CCF2-AM using
the manufacturer’s protocol (Life Technologies). Readings were recorded
with a Tecan Infinite 200 microplate reader with two filter sets with exci-
tation at 405 ± 20 nm and emission at 460 ± 40 nm or excitation at 405 ±
20 nm and emission at 530 ± 30 nm. Fluorescence micrographs were
captured with a Leica DM400B microscope.

Matchmaker Gold yeast two-hybrid screening. The Rv3354 gene
was cloned in frame with the GAL4 DNA binding domain of pGBK7. The
resultant pGBK7::Rv3354 vector was transformed into Saccharomyces
ceresuis strain Y2HGold following the manufacturer’s instructions
(Clontech). The normalized yeast two-hybrid universal human library,
fused with the GAL4 activation domain of the pGADT7 vector and stored
in the Y187 yeast strain, was purchased from Clontech. The interaction
between pGBK7::53 and pGADT7::Rv3354 served as a positive control,
whereas pGBK7::lam and pGADT7::Rv3354 were used as a control for a
negative interaction. One milliliter of the library was combined with 4 ml of
a bacterial strain mixture at 30°C for 24 h with slow shaking (50 rpm).
Yeast cells were plated on selective media (SD-Leu/-Trp), triple
(SD-His/-Leu/-Trp), and quadruple (SD-Adr/-His/-Leu/-Trp) dropout
agar plates with or without 20 mg/ml of 5-oxo-Gal and 125 mg/ml aureoba-
sidin. Colonies that turned blue were PCR amplified using the Match-
maker Insert Check PCR Mix 2 system (Clontech), and resulting products
were sequenced at the CGRB facility of Oregon State University.

Lentiviral gene transfer and coexpression. To enhance the efficient
cotransduction and expression of bacterial and host proteins in TPH-1
cells, we used the lentiviral expression system (Clontech). The coding
sequence of Rv3354 was cloned into the plLVX-drTomato-C1 (dtTomato)
vector. Homo sapiens first-strand cDNAs from total RNA were synthe-
sized using the SuperScript III first-strand synthesis system (Life Tech-
nologies). The JAMM sequence of the CSN5 gene was amplified from the
synthesized cDNA and cloned into the plLVX-ZsGreen1-C1 vector. Gen-
erated lentiviral constructs (dtTomato::Rv3354 and ZsGreen::JAMM) were
transiently transfected along with the Lenti-X H2X packaging mix (Clon-
techn) into Lenti-X 293T packaging cells to produce high-titer infectious
viral stocks. After 72 h of budding, lentiviruses were purified from the cell
supernatants and titers were determined according to the manufacturer’s
protocol (Clontech).

THP-1 cells were differentiated in 8-chamber slides, supplemented with
4 μg/ml polybrene, and infected with tdTomato::Rv3354 and Zs-
Green::JAMM simultaneously. After 24 h, cells were assessed for Tomato
and ZsGreen protein colocalization by immunofluorescence microscopy.
Coexpression of tdTomato and ZsGreen::JAMM or of dtTomato::Rv3354
and ZsGreen::JAMM (containing a mutated domain) were used as con-
trols for colocalization studies.

Comimunoprecipitation. The CSN5 protein with or without muta-
tions in the JAMM domain was expressed in the pET6×HN-C vector
(Clontech) in E. coli and purified with a His column according to the manu-
facturer’s protocol (Clontech). Alternatively, M. tuberculosis ex-
pressing Flag::Rv3354 in pMV261 was lysed by mechanical disruption.
The purified CSN5 protein was incubated with lysate of M. tuberculosis
cells expressing Rv3354. After overnight incubation at 4°C, samples were
loaded into His columns, washed, and eluted. Proteins of interest were
visualized with Western blotting using 6×HN and Flag antibodies.

Western blotting. Uninfected or WT-, 2G2-, or 2G2 (Rv3354+)–
infected THP-1 cells after 8, 24, or 48 h were lysed in Celllytic M lysis
buffer supplemented with protease inhibitor cocktail (Sigma). Samples
were centrifuged at 10,000 × g for 15 min to remove the bacterial pellet
and cell debris and then solubilized in the sample buffer (Bio-Rad). Pre-
cleared samples were separated on 12% Tris-HCl gels and transferred to
nitrocellulose. Membranes were blocked with 3% bovine serum albumin
(BSA) in PBS containing 0.1% Tween for 1 h and then incubated with
primary antibody at a 1:250 dilution for 1 h. Next, membranes were
probed with the corresponding IRDye secondary antibody (Li-Cor Bio-
sciences, Inc.) at a dilution of 1:5,000 for 30 min, and proteins were de-
tected using an Odyssey Imager (Li-Cor).

Protein kinase activity assessment. The Universal fluorometric ki-
rase assay kit (Abcam) was used to monitor the phosphotransferase ac-
tivity of the Rv3354 protein. Briefly, 5 ml of purified 6×His::Rv3354 was
dialyzed and concentrated in 3-kDa centrifugal columns (Pall). Fifty-
microliter aliquots of kinase reaction mixtures were set up with 20 μl of
ADP buffer, 25 μl of lysate/purified Rv3354/H2O, and 5 μl of 1 mM ATP
or H2O and incubated at 37°C for 30 min. Twenty microliters of the kinase
reaction mixture was combined with 20 μl of ADP sensor buffer and 10 μl
of ADP sensor and the mixture was incubated in the dark for 15 min. The
standard curve was also created and used as a reference to determine ADP
production from samples. Fluorescent readings were taken with a Tecan
reader using excitation at 530 nm/emission at 590 nm.

Statistics. Statistical analysis results are presented as means ±
standard deviations (SD) of results from two independent replicates unless
otherwise indicated. The significance level was determined by using Stu-
dent’s t test. A P value of <0.05 was considered statistically significant.

ACKNOWLEDGMENTS

We thank Martin Pavelka at the University of Rochester for providing M.
tuberculosis strain PM638.

This work was supported by the Foundation for Microbiology and
National Institutes of Health award 1R01AI47010.

REFERENCES

1. Hou JM, D’Lima NG, Rigel NW, Gibbons HS, McCann JR, Braunstein
M, Teschke CM. 2008. ATPase activity of Mycobacterium tuberculosis
SecA1 and SecA2 proteins and its importance for SecA2 function in macro-
phages. J. Bacteriol. 190:4880–4887. http://dx.doi.org/10.1128/
JB.00412-08.

2. Tan T, Lee WL, Alexander DC, Grinstein S, Liu J. 2006. The ESAT-6/
CFP-10 secretion system of Mycobacterium marinum modulates phago-
some maturation. Cell. Microbiol. 8:1417–1429. http://dx.doi.org/
10.1111/j.1462-5822.2006.00721.x.

3. Vergne I, Chu J, Lee HH, Lucas M, Belisle J, Deretic V. 2005. Mech-
anism of phagolysosome biogenesis block by viable Mycobacterium tuber-

Danelishvili et al.
Loeuillet C, Martinon F, Perez C, Munoz M, Thome M, Meylan PR. July/August 2014 Volume 5 Issue 4 e01278-14

Danelishvili L, McGarvey J, Li YJ, Bermudez LE. 2010. Secreted mycobacterial lipid is reversed by nitric oxide. Cell. Microbiol. 10.1111/j.1462-5822.2010.01147.x

Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V. 2003. Mycobacterium tuberculosis nuoG is a virulence gene that inhibits apoptosis of infected host cells. PLoS Pathog. 6:e1000283. http://dx.doi.org/10.1371/journal.ppat.1000283

Mälen H, Berven FS, Fladmark KE, Wiker HG. 2007. Comprehensive analysis of exported proteins from Mycobacterium tuberculosis H37Rv. Chem. Microbiol. 10.1111/j.1462-5822.2007.01312.x

Gomez M, Johnson S, Gennaro ML. 2007. Enhanced priming of adaptive immunity by a proapoptotic mycobacterial effector. J. Immunol. 177:1530–1545. http://dx.doi.org/10.4049/jimmunol.177.9.1530

Romagnoli A, Etna MP, Giacomini E, Pardini M, Remoli ME, Corazzari M, Etna MP, Giacomini E, Pardini M, Remoli ME, Corazzari M, Bach H, Papavinasasundaram KG, Wong D, Hmama Z, Av-Gay Y. 2006. Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. Cell 119:753–766. http://dx.doi.org/10.1016/j.cell.2004.11.038

Echalier A, Pan Y, Birol M, Tavernier N, Pintard L, Hoh F, Ebel C, Galophe N, Claret FX, Dumas C. 2013. Insights into the regulation of the human COP9 signalosome catalytic subunit, CSN5/Jab1. Proc. Natl. Acad. Sci. U. S. A. 110:1273–1278. http://dx.doi.org/10.1073/pnas.1219502110

Jin Z, Li Y, Pitti R, Lawrence D, Pham VC, Lili JR, Ashkenazi A. 2009. Culin3-based polyubiquitination and p62-dependent aggregation of caspase-8 mediate extrinsic apoptosis signaling. Cell 137:721–735. http://dx.doi.org/10.1016/j.cell.2009.03.015

Ashida H, Mimuro H, Ogawa M, Kobayashi T, Sanada T, Kim M, Sasakawa C. 2011. Cell death and infection: a double-edged sword for host and pathogen survival. J. Cell Biol. 195:931–942. http://dx.doi.org/10.1083/jcb.20108801

Lee J, Hartman M, Kornfeld H. 2009. Macrophage apoptosis in tuberculosis. Yonsei Med. J. 50:1–11. http://dx.doi.org/10.3349/ymj.2009.50.1.1

Wei N, Serino G, Deng XW. 2008. The COP9 signalosome: more than a protease. Trends Biochem. Sci. 33:592–600. http://dx.doi.org/10.1016/j.tibio.2008.09.004

Anderson DM, Frank DW. 2012. Five mechanisms of manipulation by bacterial effectors: a ubiquitous theme. PLoS Pathog. 8:e1002823. http://dx.doi.org/10.1371/journal.ppat.1002823

Angot A, Verghani A, Genis S, Peeters N. 2007. Exploitation of eukaryotic ubiquitin signaling pathways by effectors translocated by bacterial type III and type IV secretion systems. PLoS Pathog. 3:e3. http://dx.doi.org/10.1371/journal.ppat.1003000

Anderson DM, Feib JS, Monroe AL, Peterson FC, Volkman BF, Haas AL, Frank DW. 2013. Identification of the major ubiquitin-binding domain of the Pseudomonas aeruginosa ExoA2 phospholipase. J. Biol. Chem. 288:26741–26752. http://dx.doi.org/10.1074/jbc.M113.478529

Bixler S, Medina O, Welsh J, Vazquez-Torres A, Honkela M, Beck-Otschir D, Huang X, Berse M, Sperling J, Schade R, Dubiel W. 2003. Protein kinase CK2 and protein kinase D are associated with the COP9 signalosome. EMBO J. 22:1302–1312. http://dx.doi.org/10.1093/emboj/cdg127

Bek-Otschir D, Seeger M, Dubiel W. 2002. The COP9 signalosome: at the interface between signal transduction and ubiquitin-dependent proteolysis. J. Cell Sci. 115:467–473

Muthumani K, Choo AY, Premkumar A, Hwang DS, Thieu KP, Desai HA, Dey S, Merkoçi S, Bøttcher AL, Frank DW. 2008. Protein kinase E of Mycobacterium tuberculosis subverts innate immunity to evade effector response. PLoS Pathog. 4:e1001128. http://dx.doi.org/10.1371/journal.ppat.1001128

Chen JM, Zhang M, Rytkönen A, Holden DW. 2009. Identification of exported proteins from Mycobacterium tuberculosis strain H37Rv. Cell. Microbiol. 11:1702–1718. http://dx.doi.org/10.1111/j.1462-5822.2009.01312.x

Muthumani K, Choo AY, Premkumar A, Hwang DS, Thieu KP, Desai HA, Dey S, Merkoçi S, Bøttcher AL, Frank DW. 2008. Protein kinase E of Mycobacterium tuberculosis subverts innate immunity to evade effector response. PLoS Pathog. 4:e1001128. http://dx.doi.org/10.1371/journal.ppat.1001128