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Uptake of Pathogenic Intracellular Bacteria into Human and Murine Macrophages Downregulates the Eukaryotic 26S Protease Complex ATPase Gene

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A differential PCR technique detected the transcriptional downregulation of the *mss1* (mammalian suppressor of *svg1*) gene in murine J774A.1 macrophages following uptake of *Salmonella typhimurium*. This downregulation was also noted after entry of virulent strains of *Listeria monocytogenes* and *Shigella flexneri*, two other facultative intracellular bacterial species. In contrast, uptake of nonpathogenic *Escherichia coli* HB101, an *aroA* mutant of *S. typhimurium*, an invasion plasmid antigen B (*ipaB*) mutant of *S. flexneri*, hemolysin (*hly*) and positive-regulatory factor (*prfA*) mutants of *L. monocytogenes*, or latex beads produced *mss1* expression levels similar to that of uninfected macrophages. Transcriptional downregulation of *mss1* was also shown to occur in *S. typhimurium*-infected human U937 cells, albeit to an extent less than that in murine J774A.1 cells. In addition to a lower abundance of *mss1* transcripts, we also demonstrate for the first time that less MSS1 protein was detected in intracellular-bacterium-infected cells (beginning about 1 h after entry of the pathogenic intracellular bacteria) than in noninfected cells. Some strains with specific mutations in characterized genes, such as an *ipaB* mutant strain of *S. flexneri* and an *hly* mutant strain of *L. monocytogenes*, did not elicit this lower level of expression of MSS1 protein. The decrease in MSS1 within infected macrophages resulted in an accumulation of ubiquitinated proteins, substrates for MSS1. Since MSS1 comprises the ATPase part of the 26S protease that degrades ubiquitinated proteins, we hypothesize that downregulation of the *mss1* gene by intracellular bacterial entry may help subvert the host cell's normal defensive response to internalized bacteria, allowing the intracellular bacteria to survive.

Infections caused by intracellular bacterial pathogens remain a substantial world health problem. These pathogens enter into and survive within a myriad of mammalian cells, triggering a number of changes inside the host cell (6, 40). *Salmonella typhimurium*, *Listeria monocytogenes*, and *Shigella flexneri* are all facultative intracellular pathogenic species capable of entering a variety of eukaryotic cells. We have previously used the reverse transcription (RT)-PCR (44) and the differential PCR (DPCR) procedures (45, 49), the latter of which is a modification of Liang and Pardee's original work (31), to investigate transcriptional differences in murine J774A.1 macrophage cells following uptake of *L. monocytogenes* and *S. typhimurium*. One of the gene fragments that we identified previously by DPCR had very high homology (99.3%) with the gene for mitogen-activated protein kinase phosphatase 1 (MKP-1) (7). This protein regulates signal transduction events by dephosphorylating and hence inactivating mitogen-activated protein kinase (MAPK) (54). Differential transcription of this regulatory gene suggested that other genes involved in host cell regulation could be identified by DPCR technology.

To identify other host cell genes that are differentially transcribed during pathogenesis following uptake of a facultative intracellular bacterial pathogen, this study was aimed at analyzing by DPCR the responses of macrophage cells to infection by several intracellular bacterial species. These studies have uncovered a gene with high homology to the human *mss1* gene (50). The MSS1 (mammalian suppressor of *svg1*) protein has been linked to roles in cell cycle control (18, 19) and human immunodeficiency virus Tat activation (50), and it has been biochemically identified as the ATPase part of the 26S protease complex that degrades ubiquitinated proteins in the eukaryotic cell (15). This last function is particularly interesting, because ubiquitinated proteins are degraded by the 26S protease complex and some of the ubiquitinated protein substrates include oncoproteins and transcriptional regulators (11). Furthermore, the 26S protease complex is thought to have a pivotal role in regulating apoptosis, presumably either by activating a protein that facilitates cell death or by eliminating inhibitors that normally block apoptosis from occurring (20).

In this study, we have discovered for the first time the involvement of the *mss1* gene in the host cell response to the uptake of facultative intracellular bacterial pathogens, including *L. monocytogenes*, *S. flexneri*, and *S. typhimurium*. We demonstrate that entry of these bacterial species into murine macrophage cells causes a transcriptional downregulation of *mss1* that affects the protein level and ultimately leads to an accumulation of polyubiquitinated proteins in the infected eukaryotic cells. Mutants of *S. flexneri* and *L. monocytogenes* that are unable to escape from the phagosome do not affect *mss1* expression when they are used to infect macrophages. The disruption of the ubiquitin pathway by the uptake of the intracellular bacteria may have an impact on the ability of the eukaryotic cells to respond to pathogenic bacteria, undermining the host cell response.

MATERIALS AND METHODS

Bacterial strains. Wild-type *S. typhimurium* strains (LT2, C5, and TML), *Listeria monocytogenes* EGD, *S. flexneri* M90T, and *Escherichia coli* HB101 and DH5α were all obtained from the culture collection of the Laboratory of Enteric and Sexually Transmitted Diseases. An invasion plasmid antigen B (*ipaB*) mutant

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of *S. flexneri* M90T (strain SC403) originally described by High et al. (24) was provided by M. Venkatesan. Mutants of *L. monocytogenes* EGD (*hly*, *actA*, *prfA*, and *plcA*) were obtained from the culture collection of the Lehrstuhl für Mikrobiologie, Universität Würzburg, and they have been previously described (29). An *aroA* mutant strain of *S. typhimurium* LT2 (25) was provided by D. Hone. *S. typhimurium*, *S. flexneri*, and *E. coli* were grown in Luria broth (33); *L. monocytogenes* was grown in brain heart infusion broth (Difco); and the *aroA* mutant of strain LT2 was grown in Aro broth (26) at 37°C with shaking and prepared as noted previously (29). The recombinant DH5α cells transformed with the derivative of pTZ18R (Promega, Madison, Wis.) were grown on Luria agar containing 100 μg of ampicillin (Sigma Chemical Co., St. Louis, Mo.) per ml at 37°C. Mutants of *L. monocytogenes* EGD were grown in brain heart infusion broth containing 5 μg of erythromycin (Sigma) per ml. The *S. flexneri* M90T *ipaB* mutant was grown in Luria broth containing 50 μg of spectinomycin (Sigma) per ml.

Mammalian cell culture and infection. The mouse macrophage-like J774A.1 cell line and human macrophage U937 cell line were infected with bacteria according to the method of Kuhn and Goebel (29). The U937 cells were activated with phorbol 12-myristate 13-acetate (Sigma) at a concentration of 10^{-8} M (38) for 12 to 14 h and then seeded into tissue culture dishes the night before infection. A multiplicity of infection (MOI) of 25 bacteria per eukaryotic cell was used for *S. typhimurium* and *S. flexneri*, whereas an MOI of 50:1 was used for *E. coli* and *L. monocytogenes*. The addition of latex beads (0.46-μm diameter; Sigma) to macrophage cells was also done at an MOI of 50:1.

Total RNA isolation and cDNA synthesis. Noninfected and bacterium-infected J774A.1 and U937 cell total RNAs were extracted by the guanidinium thiocyanate procedure (10) at various times, and cDNAs for standard RT-PCR (6 μg) or DPCR (8 μg) were synthesized as previously described (44, 49).

PCR and processing. Standard RT-PCR (30 cycles) and limiting-dilution PCR amplifications were performed as described previously (44) with primers for β-actin, β-ACTA (5′-TGGAATCCTGTGGCATCCATGAAAC-3′) and β-ACTB (5′-TAAAACGCAGCTCAGTAACAGTCC-3′), and with primers for 32-4/62, 32-4/62A (5′-CCACCTGCATCATGGTAACTG-3′) and 32-4/62B (5′-GTGCCAAAGATGTTATTCAAGG-3′). The DPCR procedure was as outlined in the work of Schwan et al. (49) with T_{11}CA as the anchored primer and PLCA2 (5′-TGTGGAGCAG-3′) as the random primer. Products of DPCR were processed as described previously (48). RT-PCR amplifications were also performed to obtain the full-length mouse macrophage *mss1* gene open reading frame (ORF) by using the conditions previously mentioned (44) and the following primer pairs: mss1A (5′-CCATTGTGCTCTAAAGGGAAGG-3′) and 32-4/62A as well as 32-4/62C (5′-CAGTTACCATGATGCAGGTGG-3′) and 32-4/62D (5′-CAGGGTTCAGTTGTATGTCATGTA-3′).

DNA preparation and sequencing. Double-stranded plasmid DNAs were prepared with a commercial kit (Qiagen, Chatsworth, Calif.) and sequenced by the dideoxy termination procedure (42) with a Sequenase kit (United States Biochemical, Cleveland, Ohio).

Northern (RNA) blot hybridization. Ten micrograms of total RNA per lane was run on 1% denaturing agarose gels as previously noted (45). The probes for the Northern blot hybridizations were the RT-PCR products of 32-4/62 and β-actin, which were processed and used to probe the RNAs under hybridization conditions previously described (49). After blots were probed with radiolabeled 32-4/62 DNA, the filters were stripped of radioactivity with boiling 0.1% sodium dodecyl sulfate and the RNA was reprobed with the labeled β-actin RT-PCR product as described above. To determine quantitative differences between bands, filters were analyzed for densitometry with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) or the autoradiographs were scanned into the computer and processed with Adobe Photoshop software.

Nucleic acid analyses. The cloned 32-4/62 sequence was compared with sequences in the databases for nucleic acid homologies by using the BLAST program (3).

Protein extraction and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed with 10% (for ubiquitinated proteins) or 12.5% (for MSS1) separating gels (30) and 20 μg of macrophage cell total lysate prepared in cell lysis buffer (32). Proteins were electrotransferred to nylon membranes (Amersham). Membranes were blocked with blocking buffer (1% fish gelatin [Norland Products, Inc., New Brunswick, N.J.], 2% goat serum, and 0.1% bovine serum albumin [Sigma] in Tris-buffered saline containing 0.05% Tween 20 [TBS-T]) for 2 h, washed with TBS-T, and then allowed to react with either a rabbit polyclonal antibody to ubiquitinated proteins (10 μg/ml) (22) or a rabbit polyclonal antibody to mts2 that cross-reacts with MSS1 (1/1,000 dilution) (12) for 2 h at room temperature. Horseradish peroxidase-conjugated anti-rabbit antibody (Amersham) was added at a 1/10,000 dilution following three washes with TBS-T, and the membranes were incubated at room temperature for 1 h. After several more washes, bound antibodies were detected by the enhanced chemiluminescence system (Amersham). Protein autoradiographs from the enhanced chemiluminescence system were scanned into the computer, and band differences were analyzed with Adobe Photoshop software.

Nucleotide sequence accession number. The sequence of 32-4/62 has been entered into the GenBank database under accession no. U61283.

RESULTS

Detection of mRNA differences by DPCR in J774A.1 macrophages infected with *S. typhimurium*. The DPCR technique was used to identify transcriptional differences in J774A.1 macrophage cell genes arising after infection with the facultative intracellular pathogen *S. typhimurium*. Radiolabeled gene fragments were separated on 5% sequencing gels, and the resulting autoradiographs were evaluated for differences in band intensities and for the presence or absence of specific bands. From the autoradiograph shown in Fig. 1A, the DNA band marked 32-4/62 exhibited a diminished intensity in the infected lane, suggesting that downregulation of this gene had occurred. This band was processed and cloned into pTZ18R as described above.

To confirm that a transcriptional difference was indeed present and that the band difference was not an artifact of the system, the cloned 32-4/62 DNA was sequenced and primers were synthesized to run RT-PCR analyses on cDNAs from infected and noninfected macrophage cells. PCR amplifications of cDNAs from infected and noninfected J774A.1 cells with a time course of 0 h (the time point when gentamicin was added to kill extracellular bacteria) through 6 h postinfection and with the 32-4/62 sequence-specific primers allowed us to verify transcriptional downregulation of this gene and also indicated that this altered regulation occurred within 2 h of infection with the salmonellae (Fig. 1B).

Nucleic acid analysis of the 32-4/62 sequence. A search of the appropriate databases indicated that the nucleic acid sequence of 32-4/62 possesses 88% homology with part of the ORF sequence of the gene *mss1* (data not shown). The *mss1* gene was originally isolated from human HeLa cells and complements *Saccharomyces cerevisiae svg1* mutations at restrictive temperatures (50). The *mss1* cDNA was originally shown to have a size of 1.5 kb and an mRNA size of approximately 1.6 kb. Oligonucleotide primers specific for the 32-4/62 gene fragment, combined with primers located near the 5′ and 3′ ends of the reported human *mss1* cDNA clone (50), were used in RT-PCR amplifications to obtain the sequence encompassing the entire ORF of the murine macrophage homolog of human *mss1*. Predicted amino acid sequence comparisons indicated 96% homology of the murine J774A.1 macrophage cell MSS1 protein with the human HeLa cell MSS1 protein (Fig. 2). These data demonstrated that the DPCR assay had identified the mouse macrophage homolog of *mss1*.

Characterizing the specificity of *mss1* regulation with other pathogenic bacteria. Because the 32-4/62 gene is *mss1*, which is thought to be involved in the regulation of transcription within eukaryotic cells, we tested whether uptake of other pathogenic intracellular bacteria by murine macrophage cells would have any effect on *mss1* expression. Murine J774A.1 macrophage cells infected with *L. monocytogenes*, *S. flexneri*, or *S. typhimurium* all exhibited a transcriptional downregulation of *mss1* as measured by RT-PCR (Fig. 3A) and confirmed by Northern blot hybridizations (Fig. 3B). In contrast, infections of murine macrophage cells with nonpathogenic *E. coli* HB101 and an *aroA* mutant of *S. typhimurium* unable to survive within macrophage cells (25) or control infections with latex beads failed to affect the level of *mss1* expression (Fig. 3A and B). Additional strains of *S. typhimurium* (C5 and TML) were also tested by RT-PCR, and downregulation of *mss1* was similar to that observed for *S. typhimurium* LT2-infected J774A.1 cells (data not shown). The size of the 32-4/62 transcript matched the observed size of *mss1* (approximately 1.6 kb) (50). Densitometry of the Northern blot autoradiographs (Fig. 3B) revealed a 2.52 (±0.48)-fold downregulation in infected cells, similar to a

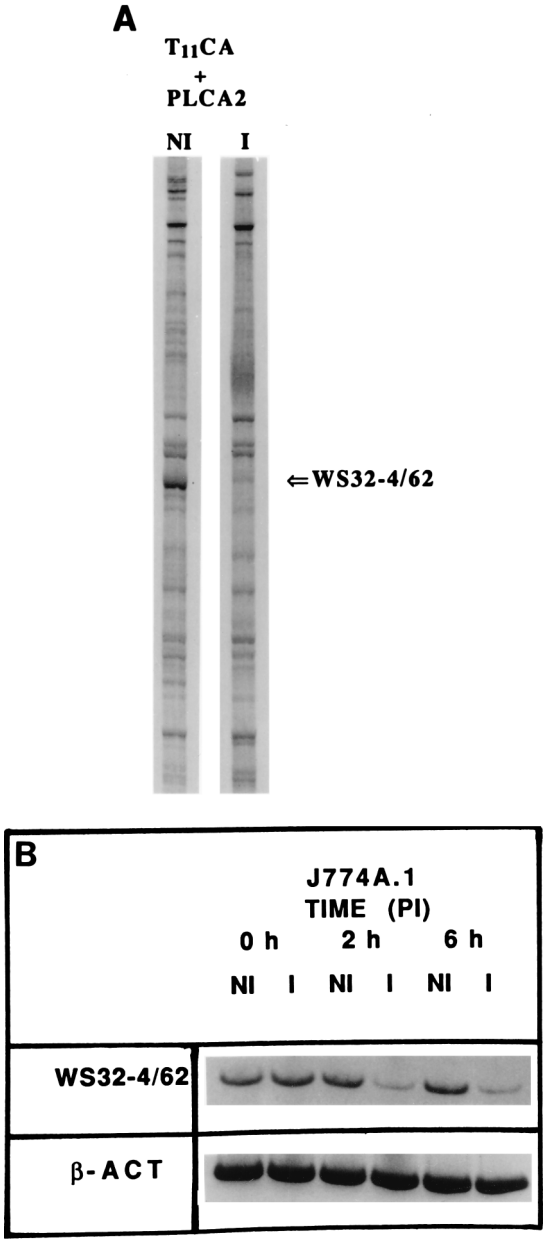

FIG. 1. (A) DPCR results. Amplifications performed with the anchored primer $T_{11}CA$ and the random primer PLCA2, comparing noninfected (NI) and *S. typhimurium*-infected (I) J774A.1 macrophage cells at 2 h postinfection. The arrow points to band WS32-4/62, exhibiting a difference on a 5% sequencing gel. (B) Confirmation of differential levels of expression of the 32-4/62 DPCR gene fragment at different time points. The mRNAs from noninfected or *S. typhimurium*-infected J774A.1 macrophage cells were tested at time points 0, 2, and 6 h postinfection. The PCR product sizes were 348 bp for β-actin (β-ACT) and 866 bp for WS32-4/62. Products were analyzed on 1.5% agarose gels, and the amplifications were conducted a minimum of three times per condition from at least two separate total RNA preparations.

2- to 4-fold downregulation found by using limiting-dilution PCR analyses (data not shown). Furthermore, this transcriptional downregulation of *mss1* was not confined to murine macrophage cells. Northern blot hybridizations with total RNAs from *S. typhimurium*-infected and noninfected human U937 macrophages also demonstrated a 1.65 (±0.11)-fold downregulation of *mss1* transcripts (Fig. 3C).

Examination of MSS1 protein levels in infected macrophages. Transcriptional regulation of a gene does not necessarily coincide with a change at the protein level. To assess whether transcriptional downregulation of *mss1* had a functional effect inside the cell, we performed immunoblot analyses on macrophage cell extracts probed with primary antibody that cross-reacts with mammalian MSS1 (12). A time course study of *S. typhimurium*-infected J774A.1 cells indicated that the level of MSS1 protein in the host cells had decreased after a 2-h infection with the salmonellae and stayed reduced through at least 6 h postinfection (Fig. 4A), similar to the results obtained from cDNA analysis (Fig. 1B). Next, J774A.1 cells were infected with a battery of pathogenic and nonpathogenic bacteria as performed before to study the transcriptional regulation of *mss1*. At 4 h of exposure, *S. flexneri*-, *S. typhimurium*-, and *L. monocytogenes*-infected J774A.1 cells showed marked reductions in their levels of host cell MSS1 (1.60-, 1.71-, and 2.51-fold decreases, respectively, as measured by densitometry) whereas infections by the nonpathogenic *aroA* mutant strain of *S. typhimurium*, *E. coli* HB101, or uptake of latex beads elicited no such diminishment (Fig. 4B). These observations demonstrate that the transcriptional regulation of *mss1* affects the amount of MSS1 protein expressed.

Effect of intracellular bacteria on the substrate of MSS1. Because the expression of MSS1 appeared to be reduced in J774A.1 cells infected with pathogenic facultative intracellular bacteria, the same macrophage cell extracts used for the experiment shown in Fig. 4B were probed with primary antibody

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HMSS1   MPDYLGADQRKTKEDEKDDKPIRALDEGDIALLKTYGQSTYSRQIKQVED -  50
        |||||||  ||||||  ||||||||||||||||||||||||  |  ||||||||||
MMMSS1  MPDYLGAEQRKTKEEEKDDKPIRALDEGDIALLKTYVQRCYSRQIKQVED

HMSS1   DIQQLLKKINELTGIKESDTGLAPPALWDLAADKQTLQSEQPLQVARCTK - 100
        ||||||||||||||||||||||||||||||||||||||||||||||||||||
MMMSS1  DIQQLLKKINELTGIKESDTGLAPPALWDLAADKQTLQSEQPLQVARCTK

HMSS1   IINADSEDPKYIINVKQFAKFVVDLSDQVAPTDIEEGMRVGVDRNKYQIH - 150
        |||||||||||||  ||||||||||||||||||||||  ||||||||||||
MMMSS1  IINADSEDPKYIIDVKQFAKFVVDLSDQVAPTDIEEGRRVGVDRNKYPIH

HMSS1   IPLPPKIDPTVTMMQVEEKPDVTYSDVGGCKEQIEKLREVVETPLLHPER - 200
        ||||||||||||||||||||||||||||||||||||||||||||||||||||
MMMSS1  IPLPPKIDPTVTMMQVEEKPDVTYSDVGGCKEQIEKLREVVETPLLHPER

HMSS1   FVNLGIEPPKGVLLFGPPGTGKTLCARAVANRTDACFIRVIGIGSELVQK - 250
        ||||||||||||||||||||||||||||||||||||||||||||||||||||
MMMSS1  FVNLGIEPPKGVLLFGPPGTGKTLCARAVANRTDACFIRVIGIGSELVQK

HMSS1   YVGEGARMVRELFEMARTKKACLIFFDEIDAIGGARFDDGAGGDNEVQRT - 300
        ||||||||||||||||||||||||||||||||||||||||||||||||||||
MMMSS1  YVGEGARMVRELFEMARTKKACLIFFDEIDAIGGARFDDGAGGDNEVQRT

HMSS1   MLELINQLDGFDPRGNIKVLMATNRPDTLDPALMRPGRLDRKIEFSLPDL - 350
        ||||||||||||||||||||||||||||||||||||||||||||||||||||
MMMSS1  MLELINQLDGFDPRGNIKVLMATNRPDTLDPALMRPGRLDRKIEFSLPDL

HMSS1   EGRTHIFKIHARSMSVERDIRFELLARLCPNSTGAEIRSVCTEAGMFAIR - 400
        ||||||||||||||||  ||  ||||||||||||||||||||||||||||||
MMMSS1  EGRTHIFKIHARSMSVQRDIGFELLARLCPNSTGAEIRSVCTEAGMFAIR

HMSS1   ARRKIATEKDFLEAVNKVIKSYAKFSATPRYMTYN* -435
        |||||||||  ||||||||||||||||  ||||||
MMMSS1  ARRKIATEKDLLEAVNKVIKSYAKFSATTRYMTYN*
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FIG. 2. Alignment of the human HeLa cell MSS1 predicted amino acid sequence (HMSS1) (7) with that of our J774A.1 murine macrophage cell MSS1 (MMMSS1). Matching amino acids are connected with a bar. Different amino acids are underlined.

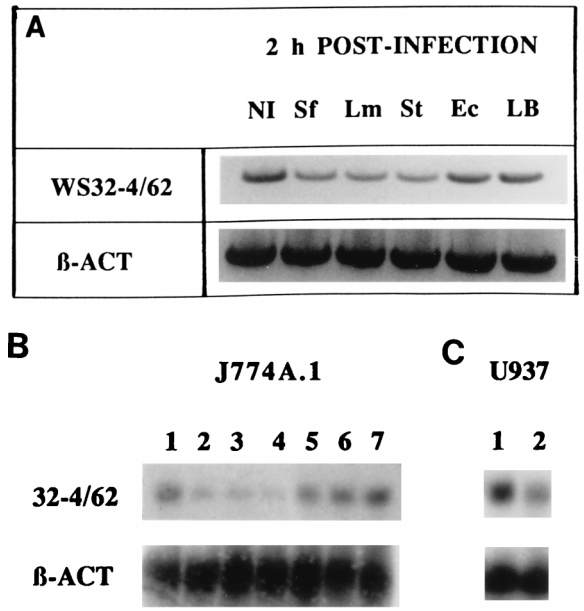

FIG. 3. Comparison of levels of 32-4/62 mRNA regulation in macrophage cells following infection with various bacterial species. (A) RT-PCR analysis of noninfected (NI) J774A.1 cells and J774A.1 cells following 2 h of infection with *S. flexneri* M90T (Sf), *L. monocytogenes* EGD (Lm), *S. typhimurium* LT2 (St), *E. coli* HB101 (Ec), or latex beads (LB). Samples were run on 1.5% agarose gels. PCR analyses were run at least three times on a minimum of two different RNA preparations for each condition listed with primers specific for 32-4/62 and β-actin (β-ACT). (B) Northern blot hybridizations of total RNAs from noninfected J774A.1 cells (lane 1) and J774A.1 cells after 2 h of infection with *S. flexneri* M90T (lane 2), *L. monocytogenes* EGD (lane 3), *S. typhimurium* LT2 (wild-type) (lane 4), *S. typhimurium* LT2 *aroA* (lane 5), *E. coli* HB101 (lane 6), or latex beads (lane 7). Transcript sizes were 1.6 kb for 32-4/62 and 2.3 kb for β-actin. (C) Northern blot hybridizations of total RNAs from noninfected U937 cells (lane 1), and U937 cells after 2 h of infection with *S. typhimurium* LT2 (lane 2). Transcript sizes were as noted in panel B.

against ubiquitinated proteins (22), substrates of the MSS1-containing 26S proteasome complex. Macrophage cells infected with pathogenic intracellular bacteria appeared to have increases in their levels of high-molecular-mass ubiquitinated proteins (>100 kDa) compared to levels in noninfected cells or macrophages infected with nonpathogenic *E. coli* HB101 (Fig. 4C), demonstrating that the transcriptional downregulation of *mss1* (Fig. 3B) and the corresponding lower level of MSS1 expression (Fig. 4A and B) also affected the ubiquitinated substrate of MSS1.

Effect of uptake of bacterial mutants on expression of MSS1 in murine macrophages. Since wild-type *L. monocytogenes* and *S. flexneri* appeared to cause downregulation of *mss1* (Fig. 3A and B), we wanted to determine which cellular event inside the macrophage cell may be responsible for this phenomenon by using mutants of each bacterial species to infect J774A.1 macrophage cells. It has previously been demonstrated that the IpaB (invasion plasmid antigen B) protein of *S. flexneri* is in part responsible for escape of these bacteria from the phagosome (24) as well as for initiating apoptosis in *S. flexneri*-infected macrophage cells (56). J774A.1 cells infected with an *ipaB* nonpolar mutant of *S. flexneri* M90T demonstrated *mss1* transcriptional levels equal to levels in noninfected cells when they were measured by RT-PCR, whereas J774A.1 cells infected with wild-type *S. flexneri* M90T showed a marked decrease in the level of *mss1* similar to what we had previously observed (Fig. 5A). Mutants of *L. monocytogenes* were also used to infect J774A.1 cells. The hemolysin (*hly*) mutant- and positive regulatory factor (*prfA*) mutant-infected macrophages did not have diminished *mss1* expression (Fig. 5B). However, other *L. monocytogenes* mutants used to infect the J774A.1 cells showed slight (*actA*7) to substantial (*plcA*) reductions in *mss1* levels more akin to that in the wild-type *L. monocytogenes*-infected macrophages. These results suggested that the listeriae needed to escape from the phagosome, since the hemolysin is necessary to dissolve the phagosomal membrane and PrfA positively regulates *hly* and other virulence factors in *L. monocytogenes*. Thus, a mutation in this gene would have a dramatic effect on hemolysin production (39).

To verify that changes at the transcriptional level were reflected in the protein level, J774A.1 cells were infected with the same battery of *L. monocytogenes* mutants used to study the transcriptional differences arising in *mss1* (Fig. 5B). Antibody that detects MSS1 protein (12) was used in immunoblots of total cell lysates of noninfected J774A.1 cells or following a 4-h infection of the macrophage cells with the various *L. monocytogenes* mutants. Decreased levels of MSS1 protein were observed in the protein lysates derived from J774A.1 cells infected with wild-type *L. monocytogenes* or a *plcA* mutant strain of *L. monocytogenes* (Fig. 5C). Mutations of *L. monocytogenes* EGD in the *hly*, *prfA*, or *actA* locus resulted in bacterial infections by these mutants that failed to cause a reduction in MSS1 levels (Fig. 5C). These results support the transcriptional data shown in Fig. 5B.

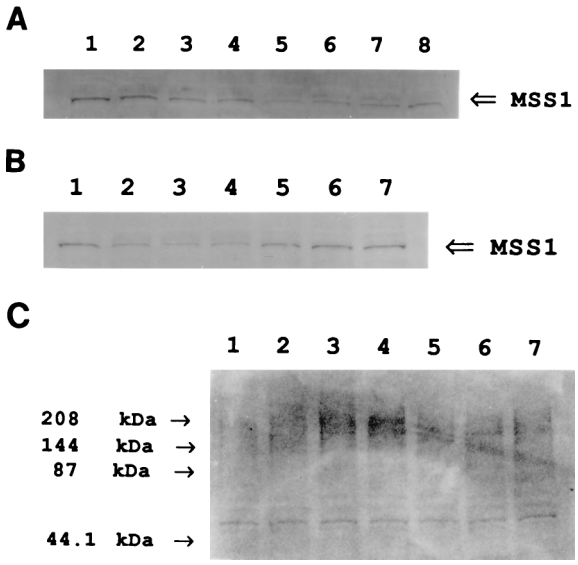

FIG. 4. Comparison of MSS1 protein expression and ubiquitinated protein levels in macrophage cells following infection with various bacterial species. (A) Time course of MSS1 expression detected by immunoblotting with antibody that cross-reacts with MSS1 protein (24). An immunoblot of protein extracts from noninfected J774A.1 cells after 15 min (lane 1) or 6 h (lane 8) of treatment and from *S. typhimurium* LT2-infected J774A.1 cells after 15 min (lane 2), 30 min (lane 3), 45 min (lane 4), 2 h (lane 5), 4 h (lane 6), or 6 h (lane 7) of elapsed time following infection is shown. (B) Comparison of levels of MSS1 expression in J774A.1 cells infected with various bacterial species. An immunoblot of protein extracts from noninfected J774A.1 cells (lane 1) and from J774A.1 cells following 4 h of infection with *S. flexneri* M90T (lane 2), *L. monocytogenes* EGD (lane 3), *S. typhimurium* LT2 (lane 4), an *S. typhimurium* LT2 *aroA* mutant (lane 5), *E. coli* HB101 (lane 6), or latex beads (lane 7) probed with anti-mts2 antibody (cross-reacts with MSS1) is shown. (C) Comparison of ubiquitinated protein levels in J774A.1 cells infected with various bacterial species. An immunoblot of protein extracts from noninfected J774A.1 cells (lane 1) and from J774A.1 cells following 4 h of infection with *S. flexneri* M90T (lane 2), *L. monocytogenes* EGD (lane 3), *S. typhimurium* LT2 (lane 4), an *S. typhimurium* LT2 *aroA* mutant (lane 5), *E. coli* HB101 (lane 6), or latex beads (lane 7) probed with anti-ubiquitinated protein antibody is shown.

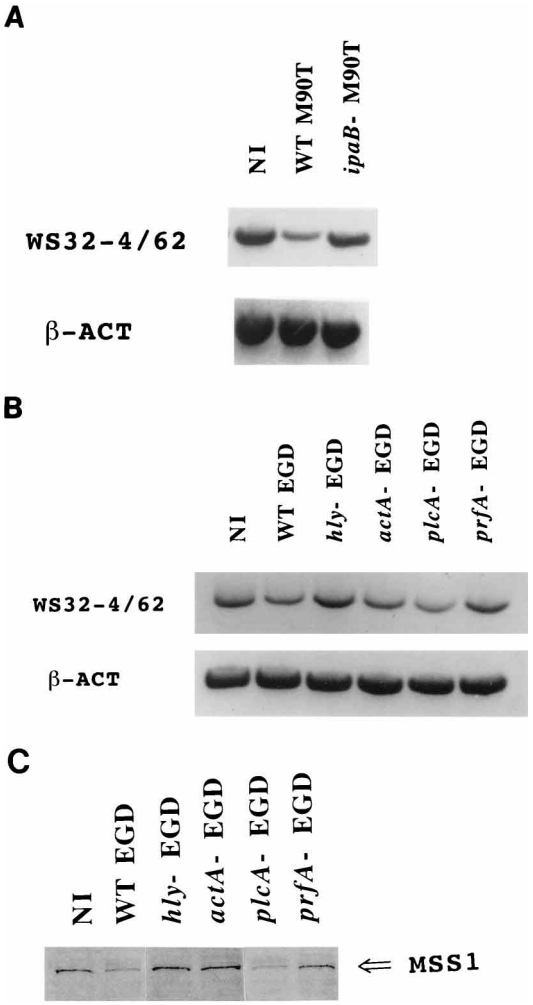

FIG. 5. Examination of *mss1* expression in J774A.1 cells infected with wild-type and mutant strains of facultative intracellular bacterial pathogens. (A) RT-PCR analysis of noninfected J774A.1 cells (NI), wild-type (WT) *S. flexneri* M90T-infected J774A.1 cells, or J774A.1 cells infected with an *ipaB* mutant strain of *S. flexneri*. β-ACT, β-actin. (B) RT-PCR analysis of noninfected J774A.1 cells compared to cells of this macrophage line infected with wild-type *L. monocytogenes* EGD or an *hly* mutant, an *actA* mutant, a *plcA* mutant, or a *prfA* mutant of strain EGD. PCR analyses were run at least twice with primers specific for 32-4/62 or β-actin, and the products were analyzed on 1.5% agarose gels. (C) Immunoblot of protein extracts from noninfected J774A.1 cells or J774A.1 cells following 4 h of infection with wild-type *L. monocytogenes* EGD or an *hly* mutant, an *actA* mutant, a *plcA* mutant, or a *prfA* mutant of strain EGD probed with anti-mts2 antibody, which cross-reacts with MSS1. The MSS1 band is noted by the arrow on the right side.

DISCUSSION

Previously, we have identified via DPCR some genes that are differentially transcribed after entry of intracellular bacteria into professional phagocytic cells (45, 49), one of which was that encoding MKP-1, which has a predicted role in regulating signal transduction events in the eukaryotic cell by dephosphorylating MAPK (54). The identification of MKP-1 suggested that other important eukaryotic genes that are differentially regulated could be characterized. Through this DPCR technique, which is a modification of the original procedure proposed by Liang and Pardee (31), differential levels of transcription of *mss1* following murine macrophage uptake of facultative intracellular pathogens has been shown for the first time in this study. This transcriptional downregulation of *mss1* was also demonstrated to affect the protein level, resulting in less MSS1 and more ubiquitinated protein expressed in pathogenic intracellular bacterium-infected macrophages.

The *mss1* gene was originally identified in human HeLa cells because of its ability to complement the mutated *sgv1* yeast gene that regulates G_1 cyclins (50), hence the name mammalian suppressor of *svg1*. A link between MSS1 and the ATPase part of the 26S protease complex was initially established genetically (19) and then confirmed biochemically (15). This 26S proteosome has multicatalytic properties that allow it to digest large proteins into small peptides and amino acids, releasing ubiquitin to allow additional rounds of proteolysis (17). Destruction of ubiquitinated proteins can influence transcription factor activity (43), and this degradation may also trigger the beginning of apoptosis (12, 20). In addition, proteasomes have been linked with the earliest stages of antigen degradation needed for cytotoxic-T-lymphocyte epitope generation (51).

The *mss1* gene appears to be a target of regulation following infection by several pathogenic intracellular bacteria that include *L. monocytogenes*, *S. typhimurium*, and *S. flexneri*. Lower expression of the MSS1 protein appears to cause an accumulation of high-molecular-mass (>100-kDa) ubiquitinated proteins in the infected macrophage cells. These high-molecular-mass ubiquitinated proteins may include the precursors of the p50 (p105) and p52 (p100) subunits of NF-κB (5), polyubiquitinated IκBα proteins (28), and other polyubiquitinated inhibitors of transcription factors. Activation of NF-κB results from two separate actions: a controlled proteolysis of the precursors of NF-κB to yield its subunits and the breakdown of the inhibitor IκB (41). Degradation of IκB has been shown to be blocked when proteosome inhibitors are used (2, 37), and it is possible that a similar outcome may result from infections by these intracellular pathogens.

Work done by Hauf et al. (23) has indicated a rapid activation of NFκB in murine macrophages following infection by virulent *L. monocytogenes*, and this activation appears to ebb after 4 h of infection. High levels of MSS1 early in the bacterial infection may allow activation of NFκB and other transcription factors by degrading their respective ubiquitinated inhibitors. However, levels of MSS1 in macrophages decrease later in the bacterial infection, reducing degradation of ubiquitinated proteins, which may include IκB, and presumably leading to less transcription factor activation. A lower level of transcription factor activation may result in a less vigorous response by the macrophage against the invading pathogenic bacteria, since activation of the transcription factors may drive some of the antibacterial responses. It is also possible that the downregulation of *mss1* may reduce the processing of bacterial antigens needed for induction of cytotoxic T lymphocytes. Sijts et al. (51) have provided clues that inhibiting proteosomes has an effect on *L. monocytogenes* antigen degradation, which presumably affects epitope presentation.

Even more intriguing is the possibility that the downregulation of *mss1* by these intracellular pathogens may be part of the apoptosis cascade in macrophage cells. The proteasome, of which MSS1 is an integral part, is involved in regulating programmed cell death in mammalian cells (20, 41). Inhibition of the proteasome function by specific proteasomal inhibitors contributes to apoptosis in human macrophage-like HL-60 cells (14). Furthermore, diminished NF-κB activation, which can result from reduced expression of *mss1* and lower levels of proteasome processing, can also be an important part of the apoptotic process (4, 53).

Previous work has shown that several facultative intracellular bacterial pathogens induce apoptosis in infected macrophage cells, as has been shown with *S. flexneri* infection of

J774A.1 cells (55) and *Legionella pneumophila* infection of HL-60 macrophage-like cells (35), and two recent studies have indicated that *S. typhimurium*-infected murine macrophage cells also undergo apoptosis following infection (8, 34). Although preliminary data suggested that *L. monocytogenes* does not contribute to apoptosis in infected macrophage cells (55), mouse dendritic cells infected with *L. monocytogenes* are triggered by listeriolysin to undergo apoptosis (21), and our own observations have indicated substantial J774A.1 cell death after an 18-h exposure to a high MOI of *L. monocytogenes* (43a). Specific bacterial products linked with eliciting apoptosis in mammalian cells include the IpaB protein from *S. flexneri* (9, 56) and listeriolysin from *L. monocytogenes* (21). Data from this study show that an *ipaB* mutant of *S. flexneri* does not affect MSS1 levels in the macrophage nor does a *hly* mutant of *L. monocytogenes*, whereas the wild-type strains of both species demonstrate marked reductions in *mss1* at both the transcriptional and protein levels (Fig. 5). In addition, *S. typhimurium* infection of murine J774A.1 cells resulted in a greater transcriptional downregulation of *mss1* than did its infection of human U937 macrophage cells. The *S. typhimurium* strain that we used in this study, LT2, is as virulent as *S. typhimurium* C5 or TML when infecting murine macrophage cells (46, 47). We have demonstrated that *S. typhimurium* entry into murine macrophage cells causes considerably more eukaryotic cell death than does entry of the same *Salmonella* serovar into human macrophage cells (47), which supports our observations regarding greater expression of *mss1* in human than in murine macrophage cells infected with *S. typhimurium*. These analyses then suggest that there may be a link between *mss1* expression in macrophages infected with virulent intracellular bacteria and the apoptosis cascade, but this association is probably not the initiating event.

These findings suggest that a broad group of intracellular bacteria are capable of regulating specific eukaryotic genes that may assist in their survival within the host cell. Pathogenic intracellular bacteria cause a variety of changes in the host cell that include transcriptional control of regulatory genes such as MKP-1 (44, 49) and the *mss1* gene reported in this study, phosphorylation of MAPK (36, 52), early activation of transcription factors (16, 23), modulation of other signal transduction proteins (1, 13, 27), upregulation of stress genes for heat shock protein 70 (Hsp70) and Hsp90 (44), and expression of various cytokines (29). Some of these intracellular changes are likely to subvert the host response to the invading bacterial threat. This cross-talk between both sides ultimately centers around survival of one or both parties, and the side that can counter the opposition more effectively may survive.

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