The Adaptor Protein p62/SQSTM1 Targets Invading Bacteria to the Autophagy Pathway

Yiyu T. Zheng, Shahab Shahnazari, Andreas Brech, Trond Lamark, Terje Johansen and John H. Brumell

J Immunol 2009; 183:5909-5916; Prepublished online 7 October 2009;
doi: 10.4049/jimmunol.0900441
http://www.jimmunol.org/content/183/9/5909

Supplementary Material
http://www.jimmunol.org/content/suppl/2009/10/06/jimmunol.0900441.DC1

References
This article cites 31 articles, 13 of which you can access for free at:
http://www.jimmunol.org/content/183/9/5909.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
The Adaptor Protein p62/SQSTM1 Targets Invading Bacteria to the Autophagy Pathway

Yiyu T. Zheng,* Shahab Shahnazari,* Andreas Brech,† Trond Lamark,‡ Terje Johansen,§ and John H. Brumell

Autophagy, a cellular degradative pathway, plays a key role in protecting the cytosol from bacterial colonization, but the mechanisms of bacterial recognition by this pathway are unclear. Autophagy is also known to degrade cargo tagged by ubiquitinated proteins, including aggregates of misfolded proteins, and peroxisomes. Autophagy of ubiquitinated cargo requires p62 (also known as SQSTM1), an adaptor protein with multiple protein-protein interaction domains, including a ubiquitin-associated (UBA) domain for ubiquitinated cargo binding and an LC3 interaction region (LIR) for binding the autophagy protein LC3. Previous studies demonstrated that the intracellular bacterial pathogen Salmonella typhimurium is targeted by autophagy during infection of host cells. Here we show that p62 is recruited to S. typhimurium targeted by autophagy, and that the recruitment of p62 is associated with ubiquitinated proteins localized to the bacteria. Expression of p62 is required for efficient autophagy of bacteria, as well as restriction of their intracellular replication. Our studies demonstrate that the surveillance of misfolded proteins and bacteria occurs via a conserved pathway, and they reveal a novel function for p62 in innate immunity. The Journal of Immunology, 2009, 183: 5909–5916.

Immune surveillance for intracellular bacteria in mammalian cells plays a crucial role in cell survival and host defense. Recently, macroautophagy (hereafter referred to as autophagy), an important cellular pathway for the degradation of long-lived proteins and damaged organelles by delivering them to the lysosome (1, 2), was shown to be involved in the innate immune response to bacteria (2, 3). Autophagy is known to target bacteria early after invasion and protect the cytosol from bacterial colonization (4, 5). However, the mechanisms by which bacteria are targeted by autophagy are not known (6).

Salmonella enterica serovar Typhimurium (S. typhimurium) is a Gram-negative bacterial pathogen that can infect a variety of hosts (7). Following S. typhimurium invasion, most bacteria reside and replicate within intracellular compartments termed Salmonella-containing vacuoles (SCVs) (8). However, it is now appreciated that a significant fraction (typically 25%) of S. typhimurium is targeted by autophagy, becoming microtubule-associated protein 1 L chain-3 positive (LC3β, an autophagy marker) (5). Autophagy was found to restrict the intracellular growth of S. typhimurium (5). Bacteria targeted by autophagy colocalize with polyubiquitinated proteins (5), suggesting ubiquitination of host/bacterial proteins may be involved in autophagy of S. typhimurium.

p62/SQSTM1 (hereafter referred to as p62) is involved in cell signaling, receptor internalization, and protein turnover (9–11). Recently, p62 was shown to directly bind ubiquitinated proteins (via its C-terminal ubiquitin-associated (UBA) domain) and LC3 (via a newly identified LC3 interacting region (LIR)) (12–15). It has been suggested that p62 acts as an adaptor by binding ubiquitinated protein aggregates and targeting them for degradation by autophagy, therefore protecting the cytosol from the toxic effects of misfolded or mutated proteins (10, 13, 14). In addition to ubiquitinated protein aggregates, peroxisomes labeled with ubiquitinated proteins were recently found to be degraded by autophagy in a p62-dependent manner (16). Therefore, p62 plays an important role in cellular homeostasis by mediating the autophagy of cargo of various sizes tagged with ubiquitinated proteins. Since S. typhimurium targeted by autophagy is often associated with ubiquitinated proteins (5), we hypothesized that p62 may play an adaptor role in the process of autophagy of bacteria.

Materials and Methods

Bacteria strains, cell culture, and pharmacologic agents

Wild-type S. typhimurium SL 1344 and bacteria expressing red fluorescence protein (RFP) (17) were used for these studies. For invasion by S. typhimurium, late-log bacterial cultures were used for infecting cells and prepared via a method optimized for bacterial invasion (18). HeLa human epithelial cells were obtained from the American Type Culture Collection. Wild-type and autophagy-deficient (Atg5−/−) mouse embryonic fibroblasts (MEFs) have been previously described (19). All cells were cultured in DMEM plus 10% FBS without antibiotics and seeded in 24-well tissue culture plates on glass coverslips 16–24 h before use.

Abbreviations used in this paper: EM, electron microscopy; LC3, microtubule-associated protein 1 light chain-3; LIR, LC3 interaction region; MEF, mouse embryonic fibroblast; p.i., postinfection; RFP, red fluorescence protein; SCV, Salmonella-containing vacuole; siRNA, small interfering RNA; SR, small interfering RNA resistant; S. typhimurium, Salmonella enterica serovar Typhimurium; Ub, ubiquitinated protein; UBA, ubiquitin associated.

Copyright © 2009 by The American Association of Immunologists, Inc. 0022-1767/09/$2.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0900441
FIGURE 1. p62 colocalizes with a population of S. typhimurium following invasion. A, HeLa cells were infected with wild-type S. typhimurium and fixed at 1 h p.i. Cells were then coimmunostained with FK2 mAb to ubiquitinated proteins (labeled with Zenon Alexa Fluor 568 mouse IgG labeling kit; see Materials and Methods), a polyclonal Ab to S. typhimurium, and a mAb to p62 as indicated. Insets show higher magnification of the boxed areas. Size bar, 10 μm. B, Cells were infected with wild-type S. typhimurium expressing RFP. Cells were fixed at the indicated time and stained with an Ab to p62. The percentage of p62+ bacteria was enumerated by fluorescence microscopy. At least 100 bacteria were counted for each time point. The average ± SD is shown for three independent experiments. C, Cells were transfected with RFP-LC3, then infected and fixed as in A; cells were then immunostained with Abs to p62 and to S. typhimurium. Insets show higher magnification of the boxed areas. Size bar, 10 μm. D, Cells were infected for 1 h, fixed, and stained for ubiquitinated proteins and/or p62. The percentage of Ub+ or Ub− bacteria colocalizing with p62 (left panel), the percentage of p62+ or p62− bacteria colocalizing with Ub (middle panel), and the percentage of GFP-LC3+ or GFP-LC3− bacteria colocalizing with p62 (right panel) were enumerated by fluorescence microscopy. At least 100 bacteria were counted for each condition. The average ± SD is shown for three independent experiments. The p values were calculated by a two-tailed Student’s t test.

Small interfering RNA (siRNA) treatment, plasmids, and transfection

p62 (no. M-010230-00) and siGenome nontargeting siRNA pool no. 2 (no. D-001206-14-20) were from Dharmacon. HeLa cells were transfected with 50 nM each siRNA 48 h before infection using Oligofectamine (Invitrogen) according to the manufacturer’s instruction. Successful p62 knockdown was confirmed by Western blot for each experiment. Plasmids GFP-LC3 (20), GFP-p62-SR (siRNA-resistant p62 tagged with GFP), and GFP-p62-SR-ΔLIR (13), or GFP-p62-SR-ΔUBA was transfected into cells using GeneJuice (Oncogene Research Products) according to the manufacturer’s instructions. To construct the GFP-SR-p62-ΔUBA plasmid pENTR-p62-SR-ΔUBA was first made by exchanging a 1219-bp Nhel-EcoRV fragment in pENTR-p62-ΔUBA (10) with a similar fragment from pENTR-p62-SR (13). Finally, GFP-p62-SR-ΔUBA was made by a Gateway LR reaction into pDestEGFP-C1 (21).

Immunofluorescence and confocal microscopy

Cells were fixed in 2.5% paraformaldehyde in PBS (pH 7.2) for 10 min at 37°C. Fixed cells were stained as previously described (18). Mouse mAb to p62 was from BD Biosciences; rabbit polyclonal Ab to p62 was from Santa Cruz Biotechnology. Rabbit polyclonal Ab to S. typhimurium (Salmonella O anti-serum group B factors 1, 4, 5, and 12) was from Difco Laboratories. FK2 mAb was from BIOMOL. For some experiments, FK2 Ab was conjugated to Alexa Fluor 568 by using the Zenon Alexa Fluor 568 mouse IgG labeling kit ( Molecular Probes). Samples were analyzed using a Zeiss Axiosvert microscope (×63 objective) and LSM 510 software. Confocal images were imported into Adobe Photoshop and assembled in Adobe Illustrator for labeling. Colocalization quantifications were performed using a Leica DMIRE2 epifluorescence microscope.

Electron microscopy (EM)

For transmission EM, cells were fixed in 2% glutaraldehyde overnight at room temperature. Samples were treated with 1% OsO4 in 0.1 M Sorenson’s phosphate buffer, stained with 1% aqueous uranyl acetate, dehydrated in a graded series of ethanol, and embedded in Epon. Samples were then sectioned, stained with 2% uranyl acetate, then 0.2% lead citrate, and examined on a FEI Tecnai 20 transmission electron microscope operating at 80 kV.

Cells infected with Salmonella were fixed for immuno-EM in 4% formyldehde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Cells were pelleted with 10% gelatin at 37°C, solidified at 4°C, and small blocks were infused with 2.3 M sucrose before mounting on specimen holders and freezing in liquid nitrogen. Sections of 70–90 nm were cut at −110°C on a Leica Ultracut and picked up with a 1:1 mixture of 2.3 M sucrose and 2% methyl cellulose on formvar/carbon-coated grids. Immunolabeling was essentially performed as described earlier (22), using mouse mAbs against p62 (BD Biosciences) and Ab to S. typhimurium (Salmonella O anti-serum group B factors 1, 4, 5, and 12; Difco Laboratories) followed by protein A-gold of 10 nm and 15 nm (CMC). We used a secondary bridging R-anti-M Ab (Dako) as intermediate after the monoclonal p62 Ab. Glutaraldehyde (0.1%) in 0.1 M PBS was used as blocking step in the double labeling procedure. Specimens were observed in a JEOL JEM-1230 at 60–80 kV and images were recorded with a digital camera (Morada), using iTEM software (both from Soft Imaging System). Further image processing was performed using Adobe Photoshop CS2 software.

Statistics

Colocalization quantifications were performed by direct visualization on a Leica DMIRE2 epifluorescence microscope. At least 100 bacteria were counted for each condition in each experiment. At least three independent experiments were performed. The means ± SD is shown in the figures, and p values were calculated using two-tailed Student’s t test for Figs. 1D, 2C, and 6B; p values for Figs. 3D, 4B, and 5B were calculated using a one-way ANOVA and Bonferroni posttests; p values for Fig. 7 were calculated using two-way ANOVA and Bonferroni posttests.

Results

To examine the localization of p62 after bacterial invasion, we infected HeLa cells with wild-type S. typhimurium and co-immunostained with an Ab to p62 and the FK2 mAb to ubiquitinated proteins. Since it is known that cytosolic bacteria are targeted by the ubiquitin system and become associated with ubiquitinated proteins, ubiquitination can be used as a marker for bacteria exposed to the cytosol (23). As shown in Fig. 1A, a population of intracellular S. typhimurium was found to colocalize with p62.
kinetic study showed that colocalization of *S. typhimurium* with p62 peaked at 1 h postinfection (p.i.) with ~35% of total intracellular bacteria associated with p62 (Fig. 1B). The p62 targeting was transient, as colocalization levels dropped to ~2% at 10 h p.i. (Fig. 1B). Similar levels of colocalization have been previously observed for a ubiquitinated protein+ (Ub+) *S. typhimurium* population (5).

Bacteria that were p62+ also labeled strongly for ubiquitinated proteins at 1 h p.i. (Fig. 1A). We observed that 82% of Ub+ *S. typhimurium* colocalized with p62, vs only ~5% for the Ub−...
population of intracellular S. typhimurium (Fig. 1D). Consistent with this, upon examination of p62+ bacteria we observed that ~96% of these colocalized with ubiquitinated proteins, vs only ~4% for p62− S. typhimurium (Fig. 1D). This suggests p62 association with bacteria is an early event and is possibly recruited via binding to ubiquitinated proteins associated with bacteria. In support of this idea, p62 association with bacteria was not observed in cells infected with ΔinAlin S. typhimurium (data not shown), a mutant strain of S. typhimurium that does not enter the cytosol after invasion, and is not associated with ubiquitinated proteins (5). Taken together, these data indicate that ubiquitinated protein binding is required for p62 association with bacteria.

Bacteria targeted by autophagy are associated with ubiquitinated proteins (5). Therefore, we examined the association of p62 with the intracellular population of bacteria targeted by autophagy. HeLa cells were transfected with RFP-LC3 and infected with wild-type S. typhimurium. LC3 is conjugated to the autophagosome membrane and is a well-characterized marker for autophagy (2, 20). Approximately 78% of LC3+ bacteria colocalized with p62, compared with only ~5% for LC3− bacteria (Fig. 1, C and D). These data demonstrate that p62 is associated with a population of S. typhimurium targeted by autophagy at an early stage after invasion.

Cells infected with S. typhimurium were analyzed by transmission EM. A population (~45%) of S. typhimurium was surrounded by multilamellar membranous structures at 1 h p.i. (Fig. 2A). These multilamellar membranes around the bacteria resembled the autophagic membranes surrounding ΔicsB Shigella flexneri (24). To determine whether the multilamellar structures are formed by autophagy, a MEF cell line lacking expression of the Atg5 gene (ΔAtg5−/−) was infected with S. typhimurium for 1 h. ΔAtg5 is essential for autophagosome formation; cells lacking Atg5 are completely deficient in autophagy, and no LC3 recruitment to bacteria is observed (5, 19). In ΔAtg5−/− MEF cells, a significantly lower fraction of bacteria was observed in multilamellar compartments, compared with wild-type MEFs (Fig. 2C). It is noteworthy that a population of bacteria (~20%) was observed in multilamellar structures in autophagy-deficient cells, indicating that bacterial virulence factors may promote the formation of these structures independently of host autophagy. Consistent with a drop in multilamellar structures around intracellular bacteria, we observed a higher fraction of bacteria within single membrane SCVs in

FIGURE 3. p62 is required for autophagy of S. typhimurium. A, A schematic diagram of different p62 constructs used in B-D and Figs. 4 and 5. B, HeLa cells were transfected with control siRNA, p62 siRNA for 48 h, or p62 siRNA plus different constructs. Protein was harvested and analyzed by Western blot using a mAb to p62. β-tubulin was used as a loading control. C, HeLa cells were transfected as in B, and also transfected with RFP-LC3. Cells were then infected with wild-type S. typhimurium and fixed at 1 h p.i. Shown are representative confocal images of cells with different constructs. Insets show higher magnification of the boxed areas. Size bar, 10 μm. D, Cells were transfected and infected as in C. The percentage of LC3+ S. typhimurium was enumerated by fluorescence microscopy. At least 100 bacteria were counted for each condition. The average ± SD is shown for three independent experiments (∗, p < 0.01 and ∗∗, p < 0.001 as determined by one-way ANOVA with Bonferroni posttest).
Atg5\(^{-/-}\) MEFs (Fig. 2B). Therefore, autophagy of *S. typhimurium* promotes the formation of multilamellar structures surrounding bacteria.

Next, we examined the localization of p62 in HeLa cells infected with *S. typhimurium* for 1 h (the time of peak autophagy and p62 localization with bacteria) by immunogold EM. As shown in Fig. 2D, p62 was observed at the multilamellar autophagy membrane surrounding *S. typhimurium*. Double-immunogold EM labeling of both *S. typhimurium* (15 nm gold particle) and p62 (10 nm gold particle) confirmed that p62 in most cases localized between the bacterial and multilamellar membranes; however, in some instances p62 also seemed to associate with the bacterial surface (Fig. 2E). Therefore, p62 is associated with bacteria targeted by autophagy.

To investigate whether p62 plays a role in the autophagy of bacteria, p62 expression was targeted by siRNA, and complemented with siRNA-resistant (SR) p62 mutant constructs (Fig. 3A). Additionally, the cells were transfected with GFP-LC3 and infected with wild-type *S. typhimurium*. The knockdown and construct expression were confirmed by Western blotting (Fig. 3B). Knockdown of endogenous p62 reduced the levels of *S. typhimurium* targeted by autophagy by \(\sim 50\%\) (Fig. 3, C and D). Therefore, p62 plays a significant role in autophagy of bacteria. As predicted, cells with p62 knockdown, and then complemented with GFP-p62-SR, showed normal autophagy of bacteria (Fig. 3, C and D). However, autophagy was not restored in p62 siRNA-treated cells expressing either GFP-p62-SR-LIR (lacking LIR domain) or GFP-p62-SR-ΔUBA (lacking UBA domain). Therefore, p62 requires both its UBA and LIR domains for its role in autophagy of *S. typhimurium*, consistent with its function as an adaptor protein.

Using this complementation strategy we examined the mechanism of targeting of p62 to bacteria. First, we focused on the recruitment of p62 to Ub\(^{+}\) bacteria. As expected, GFP-p62-SR and GFP-p62-SR-ΔLIR were recruited to Ub\(^{+}\) *S. typhimurium* in p62 siRNA-treated cells (Fig. 4), with levels of colocalization similar to endogenous p62 (Fig. 1D). However, cells complemented with GFP-p62-SR-ΔUBA showed a significant decrease in the levels of colocalization, indicating that the UBA domain is critical for the recruitment of p62 to Ub\(^{+}\) *S. typhimurium* (Fig. 4).

Next we examined recruitment of p62 to LC3\(^{+}\) bacteria. We observed that GFP-p62-SR was recruited to LC3\(^{+}\) *S. typhimurium* in p62 siRNA-treated cells (Fig. 3C), with levels of colocalization similar to endogenous p62 (Fig. 2B). This is consistent with normal levels of autophagy observed when p62 expression is complemented by the GFP-p62-SR construct (Fig. 3D). We also examined recruitment of GFP-p62-SR-ΔLIR and GFP-p62-SR-ΔUBA to LC3\(^{+}\) bacteria in p62 siRNA-treated cells. It must be borne in mind that in these cells the levels of autophagy is \(\sim 50\%\)
inhibited (Fig. 3D). The fact that autophagy is not abolished by p62 knockdown suggests the existence of an alternate pathway for regulating autophagy of bacteria. Interestingly, we observed recruitment of GFP-p62-SR-ΔUBA to LC3⁺ bacteria in p62 siRNA-treated cells (Fig. 5). In contrast, GFP-p62-SR-ΔLIR was not recruited to LC3⁺ bacteria in p62 siRNA-treated cells (Fig. 5). Therefore, p62 is recruited to Ub⁺ bacteria primarily via its UBA domain. However, p62 lacking the UBA domain can be recruited to autophagosomes containing LC3⁺ bacteria via its LIR domain. These observations provide a basis for understanding the association of p62 with both the bacterial surface and multilamellar autophagic membranes surrounding bacteria (Fig. 2D) and are consistent with the finding that p62 is recruited to autophagosomes via its LIR domain during starvation-induced autophagy (13, 15).

To further examine the role of LC3 binding in the recruitment of p62 to bacteria, we utilized Atg5⁻/- MEFs. Both wild-type and Atg5⁻/- MEFs were infected with wild-type S. typhimurium expressing RFP and immunostained for p62. Intracellular bacteria (20%) colocalized with p62 at 1 h p.i. in wild-type and Atg5⁻/- MEFs (Fig. 6). At 4 h p.i., the colocalization of p62 with S. typhimurium in wild-type MEF dropped to 10% (Fig. 6B), consistent with our observations in HeLa cells (Fig. 1B). In contrast, S. typhimurium colocalization with p62 was sustained in Atg5⁻/- MEF (Fig. 6B). This is consistent with an increase in the number of cytosolic bacteria associated with ubiquitinated proteins observed in these autophagy-deficient MEFs (5). Taken together, these findings demonstrate that p62 association with S. typhimurium can occur independently of autophagy.

Autophagy restricts the intracellular growth of bacteria (2, 5). To determine the importance of p62 in autophagy of bacteria, we assessed bacterial replication in cells where p62 expression was targeted by siRNA. We enumerated the number of intracellular S. typhimurium over a 10-h infection period using immunofluorescence and categorized as the percentage of infected cells containing a given number of intracellular bacteria (Fig. 7). Intracellular bacterial growth over the 10-h infection period was indicated by a decrease in the percentage of cells with lower numbers of bacteria and an increase in the percentage of cells with higher numbers of bacteria. At early time points, the number of bacteria per cell was similar between control siRNA and p62 siRNA-treated cells (Fig. 7). However, at 10 h p.i., p62 siRNA-transfected cells showed a significantly greater percentage of cells with high numbers of intracellular bacteria than control siRNA-treated cells (~57% vs control siRNA)
The online version of this article contains supplemental material.

Discussion

In previous studies, p62 has been found in many cellular inclusions, including Mallory bodies in chronic liver disease (25), aggresome-like induced structures induced by puromycin treatment (13), Lewy bodies in Parkinson’s disease, and neurofibrillary tangles in Alzheimer’s disease (25, 26). It has been suggested that p62 plays a cytoprotective role by providing a scaffold for misfolded or mutated proteins, initiating formation of aggregates and then targeting these for autophagic degradation (10) (Fig. 8, aggrephagy). Additionally, recent studies suggest that p62 might contribute to receptor, that mediates surveillance of multiple cargoes marked by ubiquitinated proteins, including protein aggregates, organelles, and bacteria, to regulate the role autophagy plays in cellular homeostasis and immunity.

Note added in proof. While this manuscript was in press, two studies were published that describe a similar role for p62 in xenophagy. Dupont et al. demonstrated that p62 mediates xenophagy of Shigella flexneri (2009, Cell Host Microbe, 6: 137–149). In a study of Listeria monocytogenes, Yoshikawa et al. demonstrated that a non-motile actA mutant is targeted by xenophagy through p62 recruitment (2009, Nat. Cell Biol., advanced online publication).

Acknowledgments

We thank Drs. Nat Brown, Nicola Jones, Noboru Mizushima, and Tamotsu Yoshimori for providing reagents. We thank M. Woodside and P. Paroutsis for assistance with confocal microscopy. We also thank Drs. Jorge Moscat and Gabriel Núñez for experimental advice and members of the Brunell Laboratory for constructive comments on the manuscript.

Disclosures

The authors have no financial conflicts of interest.

References


