Leishmania donovani Requires Functional Cdc42 and Rac1 To Prevent Phagosomal Maturation

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Received 20 October 2005/Returned for modification 28 November 2005/Accepted 1 February 2006

Leishmania donovani promastigotes survive inside macrophage phagosomes by inhibiting phagosomal maturation. The major surface glycoconjugate on promastigotes, lipophosphoglycan (LPG), is crucial for survival and mediates the formation of a protective shell of F-actin around the phagosome. Previous studies have demonstrated that this effect involves inhibition of protein kinase Cα. The present study shows that functional Cdc42 and Rac1 are required for the formation of F-actin around L. donovani phagosomes. Moreover, we present data showing that phagosomes containing LPG-defective L. donovani, which is unable to induce F-actin accumulation, display both elevated levels of periphagosomal F-actin and impaired phagosomal maturation in macrophages with permanently active forms of Cdc42 and Rac1. We conclude that L. donovani engages Cdc42 and Rac1 to build up a protective coat of F-actin around its phagosome to prevent phagosomal maturation.

The protozoan parasite Leishmania donovani, the causative agent of visceral leishmaniasis, is transmitted to humans through bites by infected sand flies. Flagellated Leishmania promastigotes are phagocytosed by host macrophages, where they survive inside phagosomes (9, 27). Lipophosphoglycan (LPG), which is the major surface glycoconjugate of promastigotes, is essential for intracellular survival (10, 15, 23, 30). Phagosomes containing L. donovani mutants that lack the repeating sugars of LPG (lpg2Δ/Δ knockout [KO]) proceed through the phagolysosomal pathway, and the parasites are killed (10). The mechanism by which LPG arrests phagosomal maturation in macrophages is partially clarified and includes accumulation of F-actin around newly formed phagosomes (17). The effects of LPG on actin are in part related to inhibition of protein kinase Cα (PKCα), an enzyme implicated in F-actin depolymerization at the phagosomal membrane (2, 16, 17, 23, 25).

Results from our group indicate that LPG from L. donovani interacts with GM1-enriched lipid microdomains (lipid rafts, detergent-resistant membranes) in the macrophage plasma membrane and that active PKCα colocalizes to a lesser degree with GM1 in these cells (unpublished data). Moreover, it was recently shown that LPG causes a disorganization of the phagosomal membrane, preventing normal assembly of GM1-enriched microdomains (8). In line with this, we found that disrupting membrane microdomains by cholesterol depletion, thereby mimicking the effect of LPG, raised the levels of periphagosomal F-actin around LPG-deficient lpg2Δ/Δ KO mutants. However, the levels of periphagosomal F-actin in cholesterol-depleted cells did not reach the levels observed around wild-type (WT) promastigotes (unpublished data).

The present study investigates the involvement of the Rho family members Cdc42 and Rac1 in the generation of the F-actin shell formed around phagosomes containing L. donovani promastigotes. The Rho family of small GTPases, including Rho, Rac, and Cdc42, has emerged as main regulators of the cytoskeleton (11, 14). GTP-bound Rho GTPases drive cellular processes such as cytoskeletal reorganization, migration, and proliferation (7, 14, 28). Importantly, Rho GTPases play a profound role in phagocytosis of opsonized prey (6, 22, 26, 29); however, their role in nonopsonic phagocytosis is poorly understood.

Dominant negative (N17) and constitutively active (V12) forms of Cdc42 and Rac1, expressed as fusion proteins with the human immunodeficiency virus-derived cell permeable peptide TAT, were used to study the effects of these proteins on periphagosomal F-actin and phagosomal maturation in RAW264.7 macrophages. We found that simultaneous introduction of N17Cdc42 and N17Rac1 prevented the accumulation of F-actin around phagosomes with WT L. donovani. Conversely, introduction of V12Cdc42 and V12Rac1 restored the accumulation of periphagosomal F-actin around phagosomes with LPG-defective promastigotes, resulting in inhibition of phagosomal maturation. Interestingly, introduction of N17Cdc42 and N71Rac1 into the cells, which reduced the levels of periphagosomal F-actin around WT L. donovani, did not promote maturation of these phagosomes. This result points toward a direct role for Cdc42 and Rac1 in phagosomal maturation. In conclusion, our results show that L. donovani requires functional Cdc42 and Rac1 to build up a coat of F-actin around its phagosome to prevent phagosomal maturation.

MATERIALS AND METHODS

Cells. The murine macrophage cell line RAW264.7 was cultured at 37°C and 5% CO2 in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated sterile-filtered fetal calf serum, 10 mM HEPES, pH 7.3, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Gibco BRL/Life Technologies) and used for experiment between passages 3 and 10. For microscopy, 2 × 105 cells were seeded on sterile glass coverslips placed in four-well plates and grown overnight. For flow cytometry, 5 × 105 cells were seeded in 12-well plates and grown overnight.
Parasites. WT L. donovani promastigotes and the isogenic LPG-defective mutant bg2–/– KO, both expressing green fluorescent protein (GFP), were cultured at 26°C in modified M199 medium with 50 μg/ml G418 (all from Gibco BRL/Life Technologies) as previously described (17). Expression of GFP was assessed by fluorescence microscopy. The promastigotes were identified in the stationary phase of growth. Medium was supplied the day before the experiment.

Transduction of RAW264.7 cells with TAT constructs. The pTAT-HA vector (where HA is hemagglutinin), pTAT-N17Cdc42 and pTAT-V12Rac1 were amplified from plasmids kindly gifted from Steve Dowdy. N17Rac1 and V12Rac1 were amplified from pEGFP-N17Rac1 and pEGFP-V12Rac1 using the following primers: sense, 5’-TATGCCGGTTACCA TGGCGCCACATCAAGTTGTTGTTGG3’; antisense, 5’-CTATGGCAATTCTT AGAGGAGGCCAGCGCCG-3’. The primers were designed to carry restriction sites for KpnI and EcoRI, which were used for subcloning into the pTAT-HA vector. After the correct sequences were verified, the plasmids were transformed into Escherichia coli strain BL21(DE3) (Novagen), and colonies expressing the plasmids were identified. Large-scale purifications were performed in Luria broth containing 50 μg/ml ampicillin and 0.1 mM IPTG (iso-propyl-β-D-thiogalactoside). The bacteria were collected by centrifugation (5,000 × g for 10 min at 4°C), resuspended in buffer Z (8 M urea, 10 mM imidazole), sonicated, and centrifuged (18,000 × g for 10 min at 4°C). The supernatants were loaded into Ni-nitrilotriacetic acid agarose (QIAGEN) for affinity purification of His6-tagged TAT-Cdc42 and TAT-Rac1. The columns were washed with 25 bed volumes of buffer Z before elution of the proteins with increasing concentrations of imidazole (0.1, 0.25, 0.5, and 1.0 M) in buffer Z. The eluted fractions were checked for protein concentration using Bradford reagent (Sigma), and the protein-containing fractions were pooled and loaded on a PD-10 column (Pharmacia), which had been equilibrated with a buffer containing 50 mM Tris, pH 7.4, and 1 mM EDTA. The TAT-GTPase buffer was then dialyzed overnight in the presence of 2% BSA (buffer A). The preparations were incubated for 45 min at room temperature with monoclonal rat antibodies (1DB4 diluted 1:200; Developmental Studies Hybridoma Bank, Iowa City, Iowa) against lysosome-associated membrane protein 1 (LAMP1). After being washed in buffer A, the cells were incubated for 30 min at room temperature with 200 μl/coverslip of AlexaFluor 594-conjugated goat anti-rat (Molecular Probes), diluted 1:400 in buffer A. After being washed in buffer A, the cells were mounted in DAKO Antifade (Dako). Antibody 1DB4 was replaced with purified rat immunoglobulin G to control for nonspecific labeling.

Confocal microscopy. Confocal imaging was performed in a Sararostro 2000 confocal system (Molecular Dynamics) equipped with two dual detectors and a Nikon microscope with a 60× oil immersion objective (numerical aperture, 1.4). The 488 and 514 lines of the argon laser were used for parallel activation of GFP/FITC and AlexaFluor 594, respectively. Dicoic micros with cutoff wave-lengths of 355 and 595 nm were used for the excited and emitted light, respectively. A 540DF30 nm band pass filter was used for detection of GFP/FITC, and a 605-nm long pass emission filter was used for detection of AlexaFluor 594. This filter setup ensured negligible red fluorescence in the green channel and vice versa. Internalized prey was identified by careful scanning up and down, and the horizontal section was positioned in the middle of the internalized prey. This allows (i) discrimination between attached and ingested prey and (ii) assessment of the structure of both cortical and phagosome-associated F-actin. A similar approach has been employed by others to distinguish between external (attached) and internalized particles (19, 31).

Analysis of phagocytic capacity. Following TAT treatment and infection, WT and LPG-defective parasites were treated with trypsin-EDTA at 37°C for 45 min to cleave extracellular parasites. This method has previously been used by other groups as a means for internalization of microbe-like particles. Cells were fixed with 4% paraformaldehyde and washed several times before flow cytometry was performed on a FACS Calibur (Becton Dickinson) instrument. A preset forward scatter and side scatter were used, and GFP fluorescence was analyzed in the FL-1 channel. A total of 10,000 cells per sample were counted. The population that was positive for GFP fluorescence was clearly separated from the control population, and the gates were applied accordingly. Data analysis of the percentage of the fluorescence-positive population of the total cell population (i.e., the ratio of phagocytosing cells) and the fluorescence intensity of the positive population (corresponding to the number of internalized prey) was performed. The phagocytic activity of the cells varied between the days of experiment (7 to 30% of the total cell population was found positive for trypsin-EDTA-resistant fluorescence); hence, the results were normalized to the phagocytic capacity of the WT in the individual experiments. Data are expressed as the means ± standard error of the means (SEM) of three independent experiments.

Measurement of periphagosomal and cortical F-actin. Periphagosomal F-actin was quantified from randomly scanned confocal images of AlexaFluor 594–phallacidin-labeled samples containing GFP-expressing promastigotes, as described by Holm et al. (17). In short, phagosomes containing promastigotes were identified in red/green images. Once phagosomes were identified, the fluorescence intensity profile of the positive population (corresponding to the number of internalized prey) was performed. The phagocytic capacity of the cells varied between the days of experiment (7 to 30% of the total cell population was found positive for trypsin-EDTA-resistant fluorescence); hence, the results were normalized to the phagocytic capacity of the WT in the individual experiments. Data are expressed as the means ± standard error of the means (SEM) of three independent experiments.

RESULTS AND DISCUSSION

LPG regulates the levels of periphagosomal F-actin in RAW264.7 cells. To analyze the levels of periphagosomal

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Cdc42 and Rac1 modulate F-actin around L. donovani-containing phagosomes. We used the TAT-mediated protein transduction technique (1, 4, 24, 36) to deliver dominant negative and constitutively active mutants of Cdc42 and Rac1 into RAW264.7 macrophages. The cells were transduced with a 200 nM concentration of TAT-N17Cdc42 or TAT-N17Rac1 (dominant negative mutants) or both (100 nM each). The entry of the GTPases into the cells was verified by Western blotting and an immunostaining protocol using antibodies and Fab fragments, respectively, directed toward the HA-epitope of the fusion proteins as described in Materials and Methods (data not shown). Analysis of the ratio of TAT-positive cells showed that around 80% of the population was effectively transduced (data not shown), with only slight variations between the different preparations. Several studies have shown that Cdc42 and Rac1 are required for efficient phagocytosis (6, 22, 26, 29). Therefore, we determined the capacity of cells transduced with dominant negative forms of Cdc42 and Rac1 to phagocytose unopsonized WT or LPG-defective L. donovani by using a flow cytometry-based method, as described in Materials and Methods. In this experiment, WT or LPG-defective L. donovani parasites were added to untransduced cells or cells transduced with V12Cdc42, V12Rac1, N17Cdc42, N17Rac1, or with the combined treatment of N17Cdc42 and N17Rac1. The percentage of cells positive for GFP fluorescence varied between the days of the experiments (7 to 30% of the total population) (data not shown). No significant differences in phagocytic capacity between WT or LPG-defective parasites or any of the treatments could be observed (Fig. 2A), showing that internalization occurred effectively in all treatments. This result demonstrates that, in contrast to the phagocytic process described for many opsonized microorganisms, the entry of nonopsonized L. donovani is independent of Rac1 and Cdc42.

Next, the levels of F-actin around randomly scanned individual phagosomes in the preparations were measured. We observed significantly reduced levels of F-actin around phagosomes containing WT L. donovani in cells transduced with either dominant negative Cdc42 or Rac1 compared to the phagosomes in untransduced cells (54% [N17Cdc42] and 36% [N17Rac1], respectively; P < 0.001) (Fig. 2B and C). In cells with both N17Cdc42 and N17Rac1, the levels of F-actin around phagosomes containing WT L. donovani were decreased to levels comparable to the levels observed around LPG-defective mutants (23% and 25% of the phagosomes in untransduced cells, respectively) (Fig. 2B and C). This indicates that both Cdc42 and Rac1 are required for a fully developed F-actin coat around an L. donovani-containing phagosome. During the preparation of this report, a study was published that supports our finding that Cdc42 plays a key role in the maintenance of the F-actin coat around phagosomes containing WT L. donovani (20).

Since our experiments indicated that the accumulation of F-actin around L. donovani-containing phagosomes was dependent on functional Cdc42 and Rac1, we speculated that the coat of F-actin around phagosomes containing LPG-defective mutants would be restored in cells transduced with activated Cdc42 and Rac1. Indeed, we found that introduction of either TAT-V12Cdc42 or TAT-V12Rac1 into cells prior to the addition of lpg2 KO mutants increased the levels of F-actin around phagosomes containing the parasites to levels compa-
FIG. 2. F-actin around phagosomes and cortical F-actin in cells with dominant negative or constitutively active Cdc42 and Rac1. RAW264.7 macrophages were transduced with TAT-linked N17Cdc42, N17Rac1, N17Cdc42/N17Rac1 in combination, TAT-V12Cdc42, or TAT-V12Rac1. Controls were treated with buffer. Thereafter, the cells were challenged with GFP-expressing WT L. donovani or LPG-defective mutants (lpg2 KO). (A) For flow cytometry, the cells were trypsinized and fixed after infection. The data obtained in three independent experiments represent cells positive for GFP fluorescence and are expressed as means normalized to the WT controls. (B to D) For microscopy, the cells were fixed,
rable to those measured around phagosomes with WT *L. donovani* (81% and 94% of the phagosomes in untransduced cells, respectively) (Fig. 2B and C).

To assess the effect of the transduced proteins on the overall F-actin content in cells challenged with WT or LPG-defective *L. donovani*, cortical F-actin was quantified. We observed lower levels of F-actin in cells transduced with either TAT-N17Cdc42 or TAT-N17Rac1 or with the combination of TAT-N17Cdc42/TAT-N17Rac1 and in cells challenged with *lpg*2−/− KO mutants compared to untransduced cells challenged with WT *L. donovani* (69%, 64%, 41%, and 49%, respectively; *P* < 0.001) (Fig. 2D). The levels of cortical F-actin observed in cells transduced with TAT-V12Cdc42 and challenged with the LPG-defective mutant was even higher than in untransduced cells challenged with WT *L. donovani* (21%; *P* < 0.005), but this effect was not found in cells transduced with TAT-V12Rac1 (Fig. 2D). We have previously shown that the amount of cortical F-actin in cells challenged with *lpg*2−/− KO mutants does not differ from noninfected cells, whereas WT *L. donovani* increases the amount of cortical F-actin (17). The observation that permanently activated Cdc42, but not permanently activated Rac1, increases cortical F-actin is supported by an experiment performed on human neutrophils, which shows a strong ring of cortical F-actin only in the presence of active Cdc42 (5). This suggests that cells with manipulated Cdc42 and Rac1 functions display specific traits, and thus the modulation of periphagosomal F-actin with the activated forms of these GTPases is not simply a result of increased total F-actin in the cell.

**Activation of Cdc42 and Rac1 inhibits translocation of LAMP1 to phagosomes.** Previous studies have shown that a coat of F-actin around *L. donovani* phagosomes prevents phagosomal maturation. Therefore, we investigated whether activation of Cdc42 and Rac1 would rescue the LPG-defective *lpg*2−/− KO mutant from being destroyed in a phagolysosomal compartment. We quantified the number of phagosomes positive for the late endosomal marker LAMP1 as a measure for phagosomal maturation (16). In short, the cells were transduced with TAT-V12Cdc42 or TAT-V12Rac1 and infected with the *lpg*2−/− KO mutant. The cells were thereafter fixed and stained with antibodies directed toward LAMP1 and examined in the confocal microscope. Translocation of LAMP1 to the phagosomes was classified as strong, positive, intermediate, or negative. As previously described (17), we found that translocation of LAMP1 to phagosomes containing WT *L. donovani* was significantly reduced compared to phagosomes with *lpg*2−/− KO mutants, indicating defective phagosomal maturation (*P* < 0.001). In cells transduced with TAT-V12Cdc42 or TAT-V12Rac1, translocation of LAMP1 to phagosomes with *lpg*2−/− KO mutants was reduced to the same level as with WT *L. donovani*, showing that activation of Cdc42 or Rac1 is sufficient to prevent the LPG-defective mutants from entering the phagolysosomal pathway. Interestingly, the reverse experiment using TAT-N17Cdc42 or TAT-N17Rac1 with WT *L. donovani* did not enhance the translocation of LAMP1 to WT-containing phagosomes compared to untransduced cells (Fig. 3).

The prevention of LAMP1 translocation in cells transduced with constitutively active Cdc42 or Rac1 correlates well with the accumulation of F-actin seen around the phagosomes in these cells (Fig. 2A and B). Several studies in similar and other systems have linked disassembly of periphagosomal F-actin to phagosomal maturation (2, 13, 16, 17). The exact mechanism by which the buildup of an F-actin coat around a phagosome inhibits phagosomal maturation is still not fully resolved, but it is possible that the fusion of the endosomes with the phagosome is physically prevented due to the abundant, interwoven actin filaments surrounding the phagosome (16, 17, 21, 27).

Although simultaneous inhibition of Cdc42 and Rac1 reduced the amount of periphagosomal F-actin around phagosomes containing WT *L. donovani* to levels comparable to phagosomes with LPG-defective mutants, the translocation of LAMP1 to these phagosomes was still prevented (Fig. 3). Although surprising at first glance, this result may reflect a recently identified mechanism for lysosome movement. There...
are several lines of evidence suggesting that endosomes and lysosomes move through the cytoplasm in a *Listeria*-like manner
by the formation of actin-based comet tails (3, 12, 34, 35).
In fact, a recent report showed that the formation of comet tails on endosomes is dependent on Cdc42 and the Cdc42
effector protein, Wiskott-Aldrich syndrome protein (35).
In addition, the movement of lysosomal compartments in HL60
cells is blocked by dominant negative Cdc42 but functions
normally in cells with constitutively active Cdc42 (unpublished
data). The mechanisms by which dominant negative forms of
Cdc42 and Rac1 prevent phagosomal maturation will be
the subject of future studies.
As mentioned in the introduction to this report, we had
preliminary indications that additional mechanisms besides
the inhibition of PKCα are involved in the LPG-induced buildup
of periphagosomal F-actin. Here we identified activation of
Cdc42 and Rac1 as important coplayers in *L. donovani*
pathogenesis. Our results show that LPG signals through Rac1 and Cdc42 to form a shell of F-actin around phagosomes required
for intracellular survival of *L. donovani*. The relative importance
of PKCα versus Cdc42 and Rac1 during *L. donovani*
infection needs further analysis.

ACKNOWLEDGMENTS

We are grateful to Albert Descoteaux for the *Leishmania* parasites and to Steve Dowdy for the pTAT-HA plasmids. The monoclonal
LAMP1 antibody developed by J. T. August was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices
of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological
Sciences, Iowa City, Iowa.
The project was financially supported by Swedish Medical Research Council grants 6251 (K.E.M.) and 1275 (E.S.); Swedish Research Council grants 6251 (K.E.M.) and 12725 (E.S.); Swedish Research Coun-
telasis (B.R.); and Stiftelsen Lars Hiertas Minne (B.R. and M.L.); the Swedish Society for Medical Research (B.R. and M.L.);
the Swedish Council grants 6251 (K.E.M.) and 12725 (E.S.); Swedish Research Coun-
telasis (B.R.); and Stiftelsen Lars Hiertas Minne (B.R.).

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