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The second author's surname was spelled incorrectly. The correct name is Hafid Soualhine.

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Summary

Mycobacterium tuberculosis **evades the innate antimicrobial defenses of macrophages by inhibiting the maturation of its phagosome to a bactericidal phagolysosome. Despite intense studies of the mycobacterial phagosome, the mechanism of mycobacterial persistence dependent on prolonged phagosomal retention of the coat protein coronin-1 is still unclear. The present study demonstrated that several mycobacterial proteins traffic intracellularly in *M. bovis* BCG-infected cells and that one of them, with an apparent subunit size of M_r 50,000, actively retains coronin-1 on the phagosomal membrane. This protein was initially termed coronin-interacting protein (CIP)50 and was shown to be also expressed by *M. tuberculosis* but not by the non-pathogenic species *M. smegmatis*. Cell-free system experiments using a GST-coronin-1 construct showed that binding of CIP50 to coronin-1 required cholesterol. Thereafter, mass spectrometry sequencing identified mycobacterial lipoamide dehydrogenase C (LpdC) as a coronin-1 binding protein. *M. smegmatis* over-expressing Mtb LpdC protein acquired the capacity to maintain coronin-1 on the phagosomal membrane and this prolonged its survival within the macrophage. Importantly, IFNγ-induced phagolysosome fusion in cells infected with BCG resulted in the dissociation of the LpdC-coronin-1 complex by a mechanism dependent, at least in part, on IFNγ-induced LRG-47 expression. These findings provide further support for the relevance of the LpdC-coronin-1 interaction in phagosome maturation arrest.**

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/120/16/2796/DC1

Key words: Macrophage, Phagosome biogenesis, *Mycobacterium tuberculosis*, *Mycobacterium smegmatis*, IFNγ, LRG-47

Introduction

Usually, macrophages ingesting microorganisms generate membrane-derived vacuoles called 'phagosomes' that mature progressively along the endocytic pathway leading to fusion with late endosomes and ultimately lysosomes (Desjardins et al., 1994; Vieira et al., 2002). Proper orchestration of these events leads to the destruction of the pathogen in phagolysosomes and the initiation of the appropriate innate immune response (Aderem and Ulevitch, 2000; Desjardins et al., 2005; Chan and Flynn, 2004). In striking contrast, the failure of phagosomes to fuse with lysosomes is a frequent finding following macrophage ingestion of *Mycobacterium tuberculosis* (Mtb) and the variant *M. bovis* BCG (Hestvik et al., 2005; Kusner, 2005; Deretic et al., 2006), enabling these organisms to reside in safe vacuoles that support their survival and replication. Based upon the concept that the maturation of phagosomes to phagolysosomes is critically important to the normal processing and presentation of antigens to elicit adaptive immunity, several laboratories have focused their efforts on the study of the biochemical and cell biologic aspects of mycobacterial phagosomes.

The arrest of phagosome maturation by pathogenic mycobacteria was initially defined as (1) resistance to acidification based upon phagosomal exclusion of the vesicular proton-ATPase (Xu et al., 1994; Sturgill-Koszycki et al., 1994) and (2) segregation from the endosomal-lysosomal pathway as evidenced by a weak staining for LAMP-1, CD63 and cathepsin D markers (Clemens and Horwitz, 1995). Thereafter, studies focusing on molecules interacting with the phagosomal marker Rab5, using latex bead-containing phagosomes, revealed the recruitment of EEA1 (early endosomal autoantigen 1) to Rab5 and the binding of its FYVE domain to phosphatidylinositol 3-phosphate (PtdIns3*P*) (Lawe et al., 2000). This phospholipid, which is important for normal membrane trafficking (Katzmann et al., 2002; Gruenberg and Stenmark, 2004), is generated on phagosomal membranes by the action of another Rab5 effector, hVPS34, a type III phosphatidylinositol 3-kinase (Christoforidis et al., 1999). In contrast to the situation with latex bead-containing phagosomes, on mycobacterial vacuoles the level of PtdIns3*P* was shown to be reduced and this implicated the interference of the mycobacterial phosphatidylinositol analog

lipoarabinomannan (LAM) with hVPS34 on the phagosomal membrane (Vergne et al., 2003). Additionally, within the host macrophage mycobacterium was found to secrete an enzyme (SapM), which acts as PtdIns3*P* phosphatase, thus complementing the action of LAM (Fratti et al., 2003; Vergne et al., 2005). Further studies showed that Mtb was able to block Ca^{2+} signaling and phagosome maturation in human macrophages via specific inhibition of sphingosine kinase 1 recruitment by nascent phagosomes, which normally induces Ca^{2+} signaling and phagosome maturation (Malik et al., 2003; Thompson et al., 2005).

Other investigations on the aberrant distribution of host proteins on the mycobacterial phagosome demonstrated that the actin-binding protein coronin-1 (de Hostos, 1999) (also known as p57 or TACO, for tryptophane aspartate-containing coat protein) associated transiently with normal phagosomes but remained actively retained by phagosomes containing viable mycobacteria (Ferrari et al., 1999; Gatfield and Pieters, 2000). Phagosomal association of coronin-1 depended on cholesterol, a factor shown to be essential for mycobacterial uptake by macrophages (Gatfield and Pieters, 2000). Although the mechanism of abnormal retention of coronin-1 on the phagosome was not clarified, it was proposed that this prohibited the sequential events driving phagosomes to fusion with lysosomes, ultimately contributing to the long-term survival of pathogens in host macrophages (Gatfield and Pieters, 2003).

It is likely that one or more virulence factors responsible for the retention of coronin-1 are expressed by pathogenic mycobacteria within the macrophage. Discovery of such factors would help to identify additional mechanisms responsible for the arrest of phagosome maturation and intracellular survival of mycobacteria. In the present study, we investigated mycobacterial proteins secreted into the host cell and their potential interference with phagosome maturation. The results obtained provided evidence for direct involvement of the mycobacterial protein lipoamide dehydrogenase in the prolonged retention of coronin-1 on the phagosomal membrane.

Results

M. bovis BCG and Mtb produce a 50 kDa protein that interacts with coronin-1

Previous studies have shown that coronin-1 localized around phagosomes containing BCG and Mtb and remained associated with the phagosome for a prolonged period of time (Ferrari et al., 1999; Gatfield and Pieters, 2000). By contrast, coronin-1 dissociated from phagosomes containing killed bacilli and redistributed to the sub-cortical plasma membrane indicating that retention of coronin-1 is dependent on metabolically active bacteria (Ferrari et al., 1999). Based on the observation that live mycobacteria export a variety of proteins intracellularly (Lee and Horwitz, 1995; Beatty and Russell, 2000) (supplementary material Fig. S1), we hypothesized that one or more of these proteins is a coronin-1-interacting protein. Two approaches were used to examine this possibility. First, macrophages were infected with [^{35}S]methionine-labeled mycobacteria and immunoprecipitations were performed on cell lysates with anti-coronin-1 (N-7) antibody. SDS-PAGE and autoradiography revealed a single band that corresponded to an ~50 kDa bacterial protein that associated with coronin-1 in macrophages infected with live but not killed BCG (Fig. 1A). Interestingly, this band was also absent in immunoprecipitates derived from cells infected with *M. smegmatis*. This 50 kDa mycobacterial protein was named CIP50 (for coronin-interacting protein 50 kDa). Further analyses involving competitive inhibition with recombinant coronin-1 demonstrated that N-7 antibodies specifically pulled down the 50 kDa bacterial protein associated with coronin-1 (Fig. 1B).

To seek additional evidence to support an interaction between CIP50 and coronin-1, the soluble fraction from macrophage lysates – the source of coronin-1 – was added to radioactive (^{35}S) culture filtrate proteins (CFPs) prepared from radiolabeled BCG, *M. smegmatis* or Mtb. The results shown in Fig. 1C indicate that CIP50 was detected in CFPs derived from BCG and Mtb, but not from *M. smegmatis*. These findings of an in vitro interaction are consistent with the in vivo pull-down data (Fig. 1A). Furthermore, experiments based on 2D-gel electrophoresis SDS–PAGE showed that the 50 kDa band corresponded to a single protein with an apparent pI of 5.4 (Fig. 1D). Taken together, these findings demonstrate that live, pathogenic mycobacteria express a coronin-1-interacting protein.

Interaction of CIP50 with coronin-1 is dependent on cholesterol

To explore the mechanism of the interaction between coronin-1 and CIP50, we next examined whether CIP50 could interact directly with recombinant coronin-1. Initial experiments using GST-coronin-1 fusion protein as bait to pull-down CIP50 from ^{35}S-CFPs were unsuccessful. One possibility to explain this is that expression of coronin-1 as a GST fusion protein may have altered its conformation thereby reducing its affinity for CIP50. Alternatively, additional host cell factors may be required to stabilize binding of CIP50 to coronin-1. To examine this possibility, GST-coronin-1 and ^{35}S-CFPs were mixed in the presence of coronin-1-depleted J774 lysates to provide a putative 'missing' factor(s). The results shown in Fig. 2A demonstrate that the cell lysate restored the interaction between CIP50 and coronin-1. Moreover, the active factor was resistant to heat treatment, suggesting that it was not proteinaceous. Since cholesterol is critical for coronin-1 accumulation beneath nascent mycobacterial phagosome (Gatfield and Pieters, 2000) and is heat stable, we tested whether cholesterol would substitute for cell lysate in promoting the CIP50-coronin-1 interaction. Indeed, as shown in Fig. 2A, this was the case. However, cell lysate substitution with excess of GM1 ganglioside, another abundant lipid membrane-associated molecule (Orlandi and Fishman, 1998), did not restore CIP50-coronin-1 interaction. Consistent with these findings, preincubation of cell lysate with the cholesterol-depleting agent MβCD completely inhibited this interaction. Taken together, these experiments demonstrated the specific role of cholesterol in CIP50-coronin-1 interaction.

To further examine the role of cholesterol in the interaction of CIP50 and coronin-1, J774 cells were infected with [^{35}S]methionine-labeled BCG then treated with the cholesterol-depleting agent MβCD. Lysates were prepared 4 hours later, and immunoprecipitated with N-7 mAb. The results, shown in Fig. 2B, indicate that the interaction of CIP50 with coronin-1 in vivo was significantly diminished in infected cells that were treated

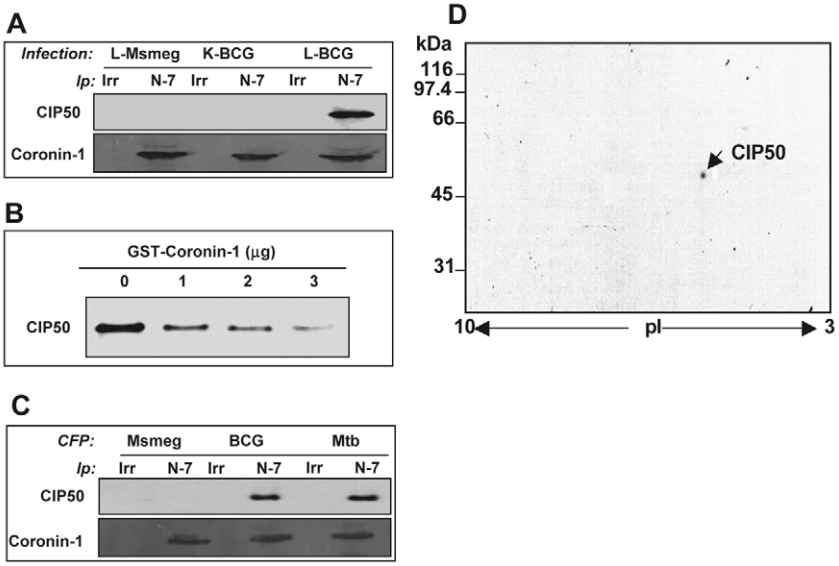

Fig. 1. Pathogenic mycobacteria express a 50 kDa coronin-1-interacting protein. (A) Macrophages were exposed to radiolabeled live (L) or gentamycin-killed (GK) BCG or live *M. smegmatis* (L-Msmeg) for 1 hour at 37°C. Partially attached, non-ingested bacteria were removed by a 5 minute treatment with trypsin-EDTA and extensive washing with HBSS and cells were replenished with culture medium and cultured at 37°C. At 4 hours post-phagocytosis cell lysates were prepared and soluble proteins were mixed with anti-coronin-1 mAb (N-7) or normal mouse IgG (Irr). (B) Cell lysates were prepared as described in A, and then mixed with increasing amounts of purified GST-coronin-1 prior to the addition of N-7 mAb. (C) ^{35}S-CFPs from *M. smegmatis*, BCG and Mtb were incubated with the soluble fraction of J774 lysates and then mixed with N-7 mAb. Material attached to the N-7 mAb was then immunoprecipitated with protein A-agarose and protein complexes were subjected to SDS-PAGE and autoradiography as described in Materials and Methods (A-C). To ensure that coronin-1 levels remained equivalent at the end of immunoprecipitation process, during the last wash of the immunoprecipitates, 10% from each treatment sample was collected and analyzed by SDS-PAGE and immunoblotting with rabbit polyclonal anti-coronin-1 and the secondary goat anti-rabbit IgG (lower panel). (D) Pulled-down CIP50 from BCG ^{35}S-CFPs was solubilized in 2D-gel rehydration solution, and analyzed by 2D-gel electrophoresis and autoradiography.

with MβCD but was restored by the addition of exogenous cholesterol. These findings demonstrate that cholesterol is involved in the stabilization of the interaction between CIP50 and coronin-1 and are consistent with previously published immunofluorescence data showing that depleting plasma membrane cholesterol specifically inhibited the retention of coronin-1 on BCG phagosomes (Gatfield and Pieters, 2000).

CIP50 is a mycobacterial lipoamide dehydrogenase

To determine the identity of CIP50, concentrated CFPs was incubated with immobilized GST-coronin-1 in the presence of 5 μM cholesterol and material eluted from the beads was separated by 2D gel analysis and subjected to LC-MS/MS as described in the Materials and Methods section. Eleven out of 16 peptide sequences matched the entry Rv0462 in the TubercuList database (http://genelist.pasteur.fr) which is dedicated to the analysis of the genomes of mycobacteria (Table 1). Rv0462 represents the mycobacterial lipoamide dehydrogenase C (LpdC; EC 1.8.1.4, 49.2 kDa and pI 5.7), an enzyme involved in part in the catalysis of the NAD^+-dependent reoxidation of lipoamide in a number of multi-enzyme complexes in Mtb and BCG (Williams, 1992; de Kok et al., 1998). LpdC is also predicted to play a similar role in *M. smegmatis* (Marcinkeviciene and Blanchard, 1997). Sequence analysis revealed 100% identity between Mtb LpdC and BCG LpdC and comparison of *M. smegmatis* LpdC with Mtb LpdC revealed an ~70% similarity (supplementary material Fig. S2).

M. smegmatis expressing Mtb LpdC maintains coronin-1 on its phagosome and displays increased intracellular survival

Several attempts to generate LpdC knock-out BCG resulted in a phenotype of attenuated growth in culture medium. Therefore, to establish a direct cause-and-effect relationship between aberrant retention of coronin-1 on BCG phagosomes and the intracellular release of LpdC, we examined whether a recombinant *M. smegmatis* strain overexpressing Mtb/BCG LpdC (rMsmeg) would reproduce the effect of BCG on coronin-1. Initial experiments using rabbit antibodies that recognize an epitope common to Mtb/BCG LpdC and *M. smegmatis* LpdC examined the extent of LpdC secretion at different stages of bacterial growth. The results obtained (Fig. 3A) showed a substantial secretion of LpdC by BCG and rMsmeg at log and stationary phases, relative to cell-associated LpdC, but limited secretion by wild-type *M. smegmatis*. Thereafter, J774 cells were infected with BCG, wild-type *M. smegmatis* and rMsmeg, and at 4 hours post-phagocytosis, cells were stained for intracellular coronin-1 and examined by confocal microscopy. It was evident that similar to BCG, and unlike wild-type *M. smegmatis*, *M. smegmatis* overexpressing LpdC retained coronin-1 on the phagosome (Fig. 3B). To examine the potential contribution of LpdC-mediated coronin-1 to bacterial survival, infected macrophages were terminated at 4 hours post-infection and the lysates were diluted and spread on 7H11 Middlebrook plates to determine the number of mycobacterial CFU remaining. Interestingly, there was an approximate tenfold increase of the rMsmeg CFUs relative to those of the parental *M. smegmatis* strain (Fig. 3C). Taken together these results demonstrated that CIP50/LpdC is, in fact, directly involved in the retention of coronin-1 on BCG phagosomes and that abnormal sequestration of coronin-1 contributes, at least in part, to prolonged survival of mycobacteria within the macrophage.

Phagosomal retention of coronin-1 is dependent on intracellular release and trafficking of CIP50/LpdC

The data presented above suggested that the interaction of CIP50/LpdC with coronin-1 is probably occurring at the cytoplasmic face of the phagosome. This suggested the

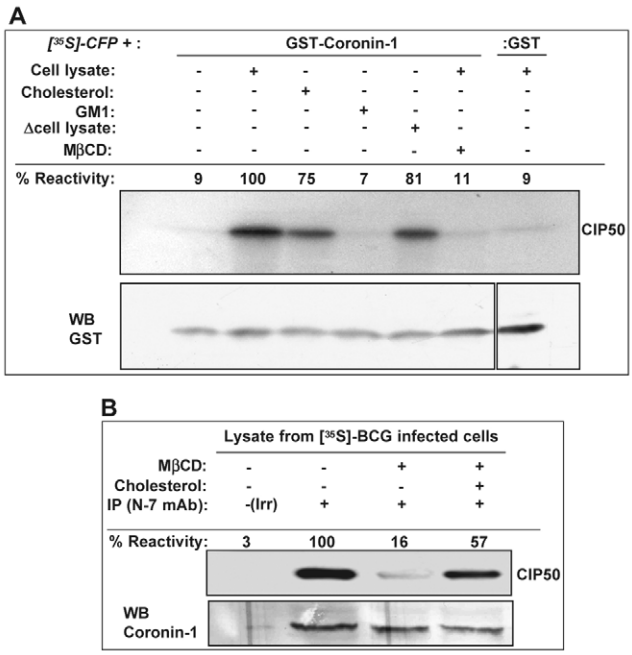

Fig. 2. Cholesterol stabilizes the binding of CIP50 to coronin-1. (A) Aliquots of ^{35}S-CFPs were mixed with agarose-immobilized GST-tagged coronin-1 (or GST alone), in the absence or in the presence of native or denatured coronin-1-depleted soluble J774 proteins (Δcell lysate), or in the presence of 1 μM cholesterol (water soluble MβCD-cholesterol complex) or 10 μM GM1 or 10 mM MβCD. The mixtures were then incubated for 2 hours at 4°C and the agarose beads were washed extensively. J774 lysates were depleted of coronin-1 by multiple cycles of immunoprecipitation with N-7 mAb. To ensure that GST-coronin-1 levels remained equivalent at the end of immunoprecipitation process, during the last wash of the immunoprecipitates, 5% from each treatment sample was collected and analyzed by SDS-PAGE and immunoblotting with anti-GST mAb and the secondary goat anti-mouse IgG (lower panel). (B) J774 cells were infected with radiolabeled BCG for 1 hour as described in Fig. 1 then 10 mM MβCD was added to the culture plate for additional 30 minutes. After extensive washing of the cells with HBSS, the culture medium was replenished with or without 1 μM cholesterol, and the cells cultured at 37°C for an additional 4 hours. Cell lysates were then prepared and subjected to immunoprecipitation with N-7 mAb. Pulled-down complexes shown in A and B were analyzed by SDS-PAGE and autoradiography. Lower panel in B shows coronin-1 western blotting of 10% immunoprecipitate from each treatment sample. Band intensities were determined by densitometry using the ImageJ software (http://rsb.info.nih.gov/ij).

hypothesis that LpdC is released within the lumen of the phagosome by Mtb, BCG and rMsmeg, but not *M. smegmatis*, and translocated to the cytosolic surface of the vacuole where it interacts with coronin-1 in cholesterol-rich domains. To examine this hypothesis, we performed a three-color confocal analysis of macrophages infected with GFP-expressing mycobacteria and stained for intracellular coronin-1 (red fluorescence) and LpdC (blue fluorescence). In cells infected with live BCG or rMsmeg, confocal images (Fig. 4A) and quantification (Fig. 4B) showed a clear and total colocalization of coronin-1 with LpdC. This provided evidence for export of LpdC to the membrane of a large majority of BCG and rMsmeg phagosomes (~56% and ~38% LpdC$^+$ phagosomes, respectively). By contrast, minimal LpdC secretion and colocalization with coronin-1 (<10% of positive phagosomes) was observed in cells infected with wild-type *M. smegmatis*. In control experiments using killed BCG, there was no detectable release of LpdC even in the vicinity of the bacteria. Consistent with these observations, EM analyses of sections of BCG-infected cells stained with anti-LpdC Ab, together with gold-labeled secondary Ab, demonstrated a trafficking of LpdC within the mycobacterial phagosome toward the cytosol (Fig. 4Ca,b). Moreover, the images obtained at high magnification (Fig. 4Cc) showed an apparent attachment of LpdC to the phagosomal membrane. No gold particles were detected in infected cells stained with normal rabbit IgG and secondary Ab (data not shown).

Translocation of LpdC to the phagosomal membrane was also examined and quantified by a FACS-based phagosome analysis previously described by our laboratory (Hmama et al., 2004). This approach uses GFP-BCG (FL1, green

Table 1. Mass spectrometry analysis of the mycobacterial coronin-1-interacting protein

Peptide	Name	Best confidence	Bin score
AHGVGDPSGFVK	Lipoamide dehydrogenase (Mtb)	90	34
AIGVDDYMR	Lipoamide dehydrogenase (Mtb)	75	20
ALPNEDADVSK	Lipoamide dehydrogenase (Mtb)	75	20
ATFJQPNVASFGLTEQQAR	Lipoamide dehydrogenase (Mtb)	99	36
DGVAQELKAEK	Lipoamide dehydrogenase (Mtb)	99	36
HGELLGGHLVGHDVAELLPELTLAQR	Lipoamide dehydrogenase (Mtb)	75	24
LGVTILTATK	Lipoamide dehydrogenase (Mtb)	99	46
LVPGTSLSANVVTYEEQILSR	Lipoamide dehydrogenase (Mtb)	75	20
NAELVHIFTK	Lipoamide dehydrogenase (Mtb)	99	54
NYGVDVTIVEFLPR	Lipoamide dehydrogenase (Mtb)	99	40
VESIADGGSQVTVTVTK	Lipoamide dehydrogenase (Mtb)	50	18
AGAATEVELKER	groEL protein 2 (Mtb)	75	24
TTLTAAITK	Elongation factor Tu (Mtb)	75	20
EQHNVLNASFEK	Lactoferrin binding protein A precursor (Neisseria meningitidis)	90	28
AEFVEVTK	Serum albumin precursor (Bos taurus)	99	47
ELTTEIDNNIEQISSYK	Keratin 10 (Homo sapiens)	50	16

All peptides listed in this table are significant matches for Rv0462 based on the best confidence and bin scores calculated by the ABI Pro QUANT MS software (www.appliedbiosystems.com).

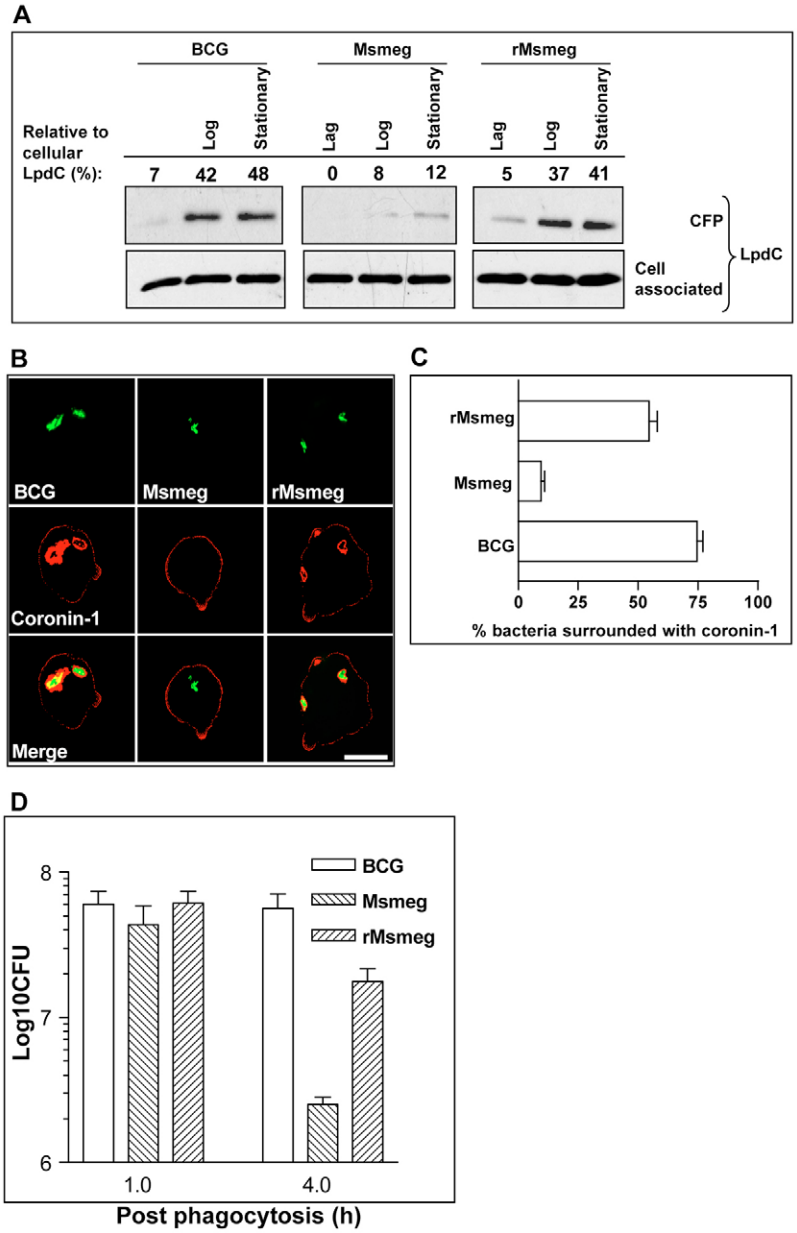

Fig. 3. *M. smegmatis* overexpressing Mtb LpdC retains coronin-1 on its phagosome and survives better within the macrophage. (A) BCG, *M. smegmatis* (Msmeg) and recombinant *M. smegmatis* expressing Mtb LpdC (rMsmeg) grown to lag, log and stationary phase in 7H9-OADC were pelleted and cultured (16-18 hours for BCG and 3-4 hours for *M. smegmatis*) in modified Dubos medium. CFPs corresponding to ~108 CFU were 10% TCA precipitated and the culture pellets were lysed by sonication in 75 mM Tris pH 8.8 plus 4 mM EDTA and 100 mM NaCl. CFPs and soluble fractions of bacterial lysates were resolved by SDS-PAGE followed by immunoblotting with anti-LpdC antibodies. (B) J774 cells adhering to glass coverslips were infected with GFP-BCG, GFP-smegmatis (Msmeg) or recombinant M. smegmatis expressing Mtb LpdC and GFP (rMsmeg). At 4 hours post-phagocytosis, cells were fixed, permeabilized and stained with rabbit anti-coronin-1 as described previously (Sendide et al., 2005a). Samples were analyzed by digital confocal microscopy using an epifluorescence microscope. The yellow signal indicates colocalization of green (bacteria) and red (coronin) fluorescence. (C) Quantification of bacterial phagosomes surrounded with coronin-1 observed in 15-20 cells from three individual experiments. (D) Macrophages were infected with the indicated bacteria and at 1 and 4 hours post-phagocytosis, bacteria were released by treatment with 0.1% Triton X-100 in PBS. Thereafter serial dilutions were prepared in PBS and plated on 7H11 plates in triplicate as described previously (Sendide et al., 2004). The data are presented as mean ± s.d. of CFU counts obtained from two independent experiments.

fluorescence) and PKH26-labeled host cell (FL2, red-fluorescent cell surface) to induce the formation of red-fluorescent phagosomes containing green-fluorescent bacteria. The specific optical properties (FL1 and FL2) of phagosomes in crude preparations allow their identification by flow cytometry without the need for extensive purification and isolation from cell debris and other organelles (supplementary material Fig. S3). Crude phagosomes were prepared at 4 hours post-phagocytosis and surface stained with normal rabbit IgG (control) or antibodies to either coronin-1 or LpdC. Immunostaining was then revealed with Alexa Fluor 647-conjugated goat anti-rabbit IgG (FL3, blue fluorescence) and the expression of coronin-1 and LpdC proteins on the phagosome surface (double red- and green-positive events) were quantified by FACS. Consistent with the confocal and EM data, the fluorescence histograms in Fig. 4D showed a high frequency of LpdC staining in phagosomes containing live BCG (~52%) or rMsmeg (~33%), which matched the frequency of coronin-1 signal (~73% for live BCG and ~48% for rMsmeg). By contrast, no LpdC signal was detected in preparations derived from killed BCG or wild-type *M. smegmatis*, which contained few (~10%) coronin-1$^+$ phagosomes. Taken together, these results demonstrate that, within the host cell, BCG and rMsmeg export LpdC for interaction with and retention of coronin-1 on the phagosomal membrane. whereas the non-pathogen *M. smegmatis*, fails to retain coronin-1 on its phagosome.

The interaction of coronin-1 with CIP50/LpdC in IFNγ activated macrophages

IFNγ is a key cytokine that stimulates infected host macrophages to directly inhibit the replication of mycobacteria (Dalton et al., 1993). This property of IFNγ appears to be dependent, at least in part, on its capacity to promote the

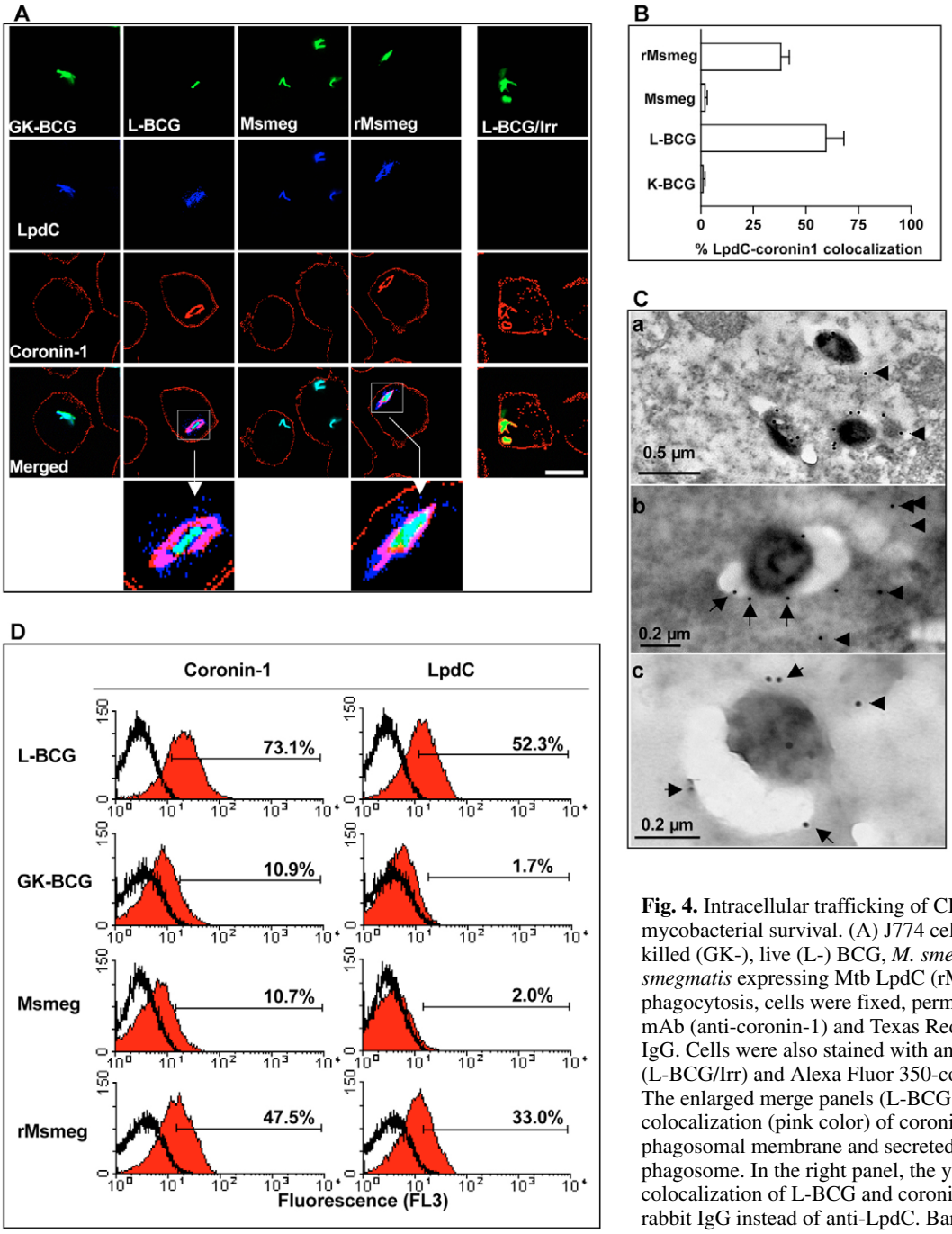

Fig. 4. Intracellular trafficking of CIP50/LpdC and its role in mycobacterial survival. (A) J774 cells were infected with gentamycin killed (GK-), live (L-) BCG, *M. smegmatis* (Msmeg) or *M. smegmatis* expressing Mtb LpdC (rMsmeg). At 4 hours post-phagocytosis, cells were fixed, permeabilized and stained with N-7 mAb (anti-coronin-1) and Texas Red-conjugated goat anti-mouse IgG. Cells were also stained with anti-LpdC or normal rabbit serum (L-BCG/Irr) and Alexa Fluor 350-conjugated goat anti-rabbit IgG. The enlarged merge panels (L-BCG and rMsmeg), show colocalization (pink color) of coronin-1 (red) with LpdC (blue) in the phagosomal membrane and secreted LpdC (blue) surrounding the phagosome. In the right panel, the yellow signal indicates colocalization of L-BCG and coronin-1 in cells stained with normal rabbit IgG instead of anti-LpdC. Bar, 10 µm. (B) Quantification of LpdC-coronin-1 colocalization in phagosomes observed in 20-25 cells from two individual experiments. (C) Thin sections of BCG-infected cells were fixed with paraformaldehyde and glutaraldehyde and incubated sequentially with rabbit anti-LpdC IgG and gold-conjugated goat anti-rabbit IgG to visualize LpdC (15 nm gold). EM grids were examined with a Tecnai 12 electron microscope at magnification of 37000× (a), 59000× (b) and 97000× (c). The arrows indicate gold particles localized on the phagosomal membrane and the arrowheads indicate gold particles localized in the cytosol. (D) PKH26-stained cells (10^7/culture plate) were allowed to ingest mycobacteria and phagosomes were prepared at 4 hours post-phagocytosis. Phagosome preparations were stained with normal rabbit IgG or specific antibodies to coronin-1 or LpdC. Preparations were washed and stained with Alexa Fluor 647-labeled secondary antibodies. After adequate compensations, blue fluorescence was analyzed on double (red and green fluorescent)-positive events, which are green fluorescent bacteria surrounded by a red phagosomal membrane (supplementary material Fig. S3); results are expressed as histograms. In each panel, the left histogram represents phagosomes stained with specific antibodies, and the histogram displaced to the right (red) represents phagosomes stained with normal IgG. Values represent the proportion of phagosomes expressing coronin-1 or LpdC.

maturation of the mycobacterial phagosome and intracellular killing (Via et al., 1998; Hmama et al., 2004; Gutierrez et al., 2004). We therefore examined the CIP50-coronin-1 interaction in the context of IFNγ-induced phagosome maturation. Initial

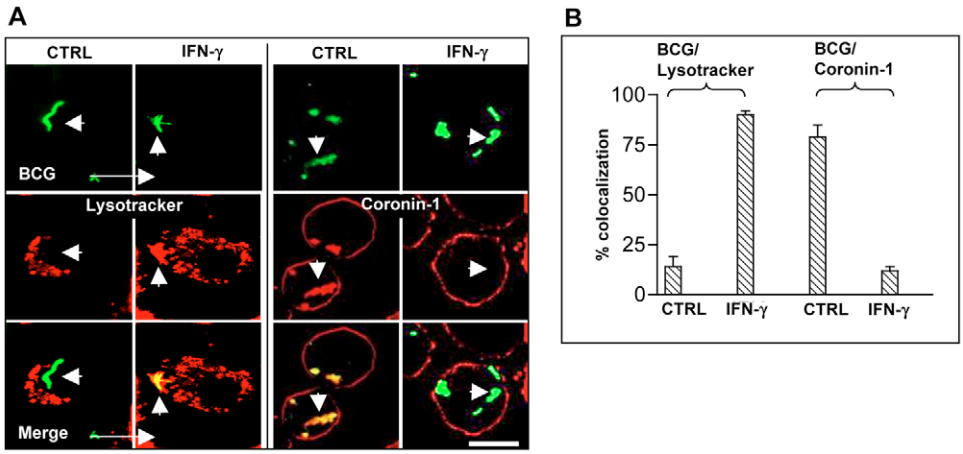

Fig. 5. IFNγ disrupts CIP50-coronin-1 interaction. (A) Left panel: control and IFNγ-stimulated (200 U/ml, 24 hours at 37°C) J774 cells were washed and reincubated in the presence of 50 nM LysoTracker Red DND 99 for 2 hours. After three washes with HBSS, cells were then infected with GFP-BCG and chased for 4 hours. Right panel: control and IFNγ-treated cells were infected with GFP-BCG and stained for coronin-1 as described in Fig. 1. (B) Quantification of BCG-LysoTracker and BCG-coronin-1 colocalizations in phagosomes observed in 15-20 cells from three individual experiments.

experiments examined phagosome maturation in J774 cells stimulated with IFNγ and loaded with LysoTracker Red, an acidotropic fluorescent dye that accumulates in acidic organelles (Via et al., 1998). As expected, the confocal images obtained showed increased colocalization of LysoTracker containing organelles with BCG phagosomes in IFNγ-treated cells suggestive of phagolysosome fusion (Fig. 5). Parallel experiments of intracellular staining demonstrated that IFNγ-induced phagolysosome fusion is concomitant to the release of coronin-1 from ~90% mycobacterial phagosome (Fig. 5). These data demonstrate that aberrant retention of the phagosomal coat protein coronin-1 is associated with arrest of mycobacterial phagosome maturation.

LRG-47 expression inhibits coronin-1 retention on mycobacterial phagosome

LRG-47 is a novel GTPase that has been recently identified as a down-stream effector of IFNγ that promotes phagosome maturation and intracellular killing of mycobacteria (MacMicking et al., 2003; Gutierrez et al., 2004). To investigate whether LRG-47 is involved in mediating IFNγ-induced coronin-1 dissociation from mycobacterial phagosome, we first examined the extent of LRG-47 protein expression in J774 cells. We found that LRG-47 was expressed minimally in resting cells, but was strongly upregulated after 24 hours stimulation with IFNγ (Fig. 6A). Next, J774 cells were transfected with GFP-LRG-47 or vector expressing GFP alone (control) and infected with BCG expressing blue fluorescent protein (BFP-BCG). Phagosomes were then examined at 4 hours post-phagocytosis by confocal microscopy. As expected, a large majority of mycobacterial phagosomes in cells transfected with GFP alone resisted fusion with LysoTracker-rich lysosomes and maintained coronin-1 on their membranes (Fig. 6B). However, the block of acidification of phagosomes in infected cells was largely overcome in LRG-47-transfected cells and coincided with the release of coronin-1 from phagosomal membranes. Moreover, expression of LRG-47, as well as IFNγ stimulation, blocked intracellular interaction of coronin-1 with CIP50/LpdC (Fig. 6C, upper panel). Control western blotting experiments with anti-coronin-1 antibodies confirmed that disruption of LpdC-coronin-1 interaction in cells stimulated with IFNγ or transfected with LRG-47 was not due to a decrease of coronin-1 levels (Fig. 6D, lower panel). Taken together, these data demonstrated that LRG-47 recruitment to the phagosomal membrane promoted phagosome maturation, and that this was associated with the release of coronin-1 from the phagosomal membrane.

Discussion

Coronins are a family of WD repeat proteins that are defined as F-actin-associated proteins widely expressed in eukaryotic cells (de Hostos, 1999). The mammalian isoform coronin-1 (also known as p57 or TACO) is expressed exclusively by leukocytes (Suzuki et al., 1995; Ferrari et al., 1999) suggesting a cell type-specific function. In this context, recent study using coronin $1^{-/-}$ mice revealed an important role of coronin-1 in actin dynamics regulating T lymphocyte trafficking and homeostasis (Foger et al., 2006). In phagocytic cells, phagocytosis and phagosome closure were shown to be depressed in coronin mutant cells (Maniak et al., 1995; Yan et al., 2005). Additionally, based upon the localization of coronin-1 beneath the cell membrane, several independent reports provided clear evidence that coronin-1 is an important regulator of the events that transpire during the early stages of phagosome biogenesis (Grogan et al., 1997; Yan et al., 2005). Indeed, abnormal retention of coronin-1 on the phagosome was shown to correlate with a block of fusion with lysosomes in macrophage infected with *M. bovis* BCG (Ferrari et al., 1999; Gatfield and Pieters, 2000) and *Helicobacter pylori* (Zheng and Jones, 2003).

To address the mechanism responsible for the retention of coronin-1 on the BCG phagosome, we hypothesized that mycobacterial proteins exported into the phagosomal lumen may interfere with various macrophage functions, including phagosome biogenesis. Indeed, pull-down experiments

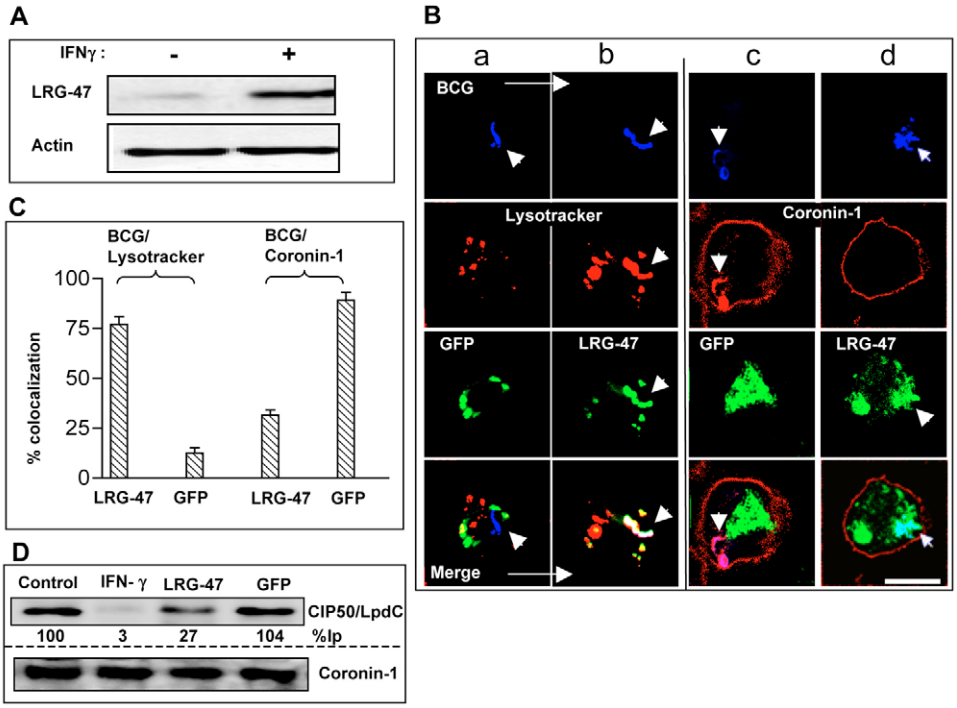

Fig. 6. Intracellular expression of LRG-47 dislocates coronin-1 from mycobacterial phagosomes. (A) Lysates from control and IFNγ-stimulated J774 cells were examined by SDS-PAGE and immunoblotting with anti-LRG-47 antibodies, then membranes were stripped and reprobed with anti-actin antibodies. (B) J774 cells were transfected with GFP or GFP-LRG-47 as described in Materials and Methods. In a first series of experiments (a,b), cells were preloaded with LysoTracker Red and infected with BCG expressing *Ebfp* (BFP-BCG) then fixed at 4 hours post-phagocytosis. In a second series of experiments (c and d), transfected cells were directly infected with BFP-BCG and stained with coronin-1 at 4 hours post-phagocytosis. In b, the white signal indicates simultaneous colocalization of BCG vacuoles with LysoTraker and LRG-47. In c, the magenta signal indicates colocalization of BCG with coronin-1. The cyan signal in d indicates colocalization of BCG phagosome with LRG-47. Short arrows indicate the position of BCG bacilli. (C) Quantification of BCG-LysoTracker and BCG-coronin-1 colocalizations in phagosomes observed in 15-20 cells from two individual experiments. (D) Upper panel: control, IFNγ-stimulated, GFP- or GFP-LRG-47-transfected cells were infected with radiolabeled BCG and at 4 hours post-phagocytosis, intracellular CIP50-coronin-1 interaction was examined as described in Fig. 1. The relative amounts of CIP50 co-immunoprecipitated with coronin-1 were determined by radioactive count of small aliquots of immunoprecipitated material. Lower panel: total cell lysate form the same treatments were also examined for expression of coronin-1 by western blotting with anti-coronin-1 antibodies.

demonstrated that a mycobacterial protein secreted intracellularly (thereafter named CIP50) bound to coronin-1 and this correlated with prolonged retention of coronin-1 on phagosomes containing live but not killed BCG. Of particular note, was the detection of a coronin-1 binding partner in BCG and Mtb culture supernatants but not in that of the non-pathogenic *M. smegmatis*. These findings are consistent with the failure of *M. smegmatis* both to retain coronin-1 on the phagosome and to survive within the macrophage (Via et al., 1998; Sendide et al., 2004).

An important result emanating from the present study was the identification of LpdC as a mycobacterial protein binding partner for coronin-1. In Mtb and BCG, LpdC is the E3 component of the pyruvate dehydrogenase complex and it also forms an integral component of peroxynitrite reductase/peroxidase, which was recently reported to be crucial for mycobacterial antioxidant defenses (Bryk et al., 2002; Rhee et al., 2005). Moreover, the in vivo relevance of LpdC as a potential bacterial virulence factor was demonstrated in *Pneumococcus* infection models where the lack of LpdC increased the sensitivity of the bacterium to oxidative stress and attenuated its survival in mice (Smith et al., 2002). These observations suggest the possibility that expression of LpdC in the phagosome might protect mycobacteria from the rapid production of reactive oxygen species, one of the crucial initial defense mechanisms of the host macrophage in response to ingested pathogens (Babior, 1995).

LpdC does not contain a transmembrane domain or peptide signal and thus its export into phagosomes indicated that there may be an alternate secretion system yet to be defined. Other mycobacterial proteins such as glutamine synthetase (Harth and Horwitz, 1997), superoxide dismutase (Harth and Horwitz, 1999) and mycobacterial tyrosine phosphatase (Koul et al., 2000) are also secreted into the extracellular medium in the absence of signal peptides at the N terminus and the mechanism employed in exporting these proteins is presently unclear.

A previous study by Gatfield and Pieters showed that cholesterol accumulated at the site of mycobacterial entry, and depleting plasma cholesterol specifically inhibited phagocytosis (Gatfield and Pieters, 2000). A question that remained unanswered was: beyond influencing mycobacterial uptake, what was the impact of cholesterol on phagosome biogenesis? Results from the present study shed light on this issue in that they show: (i) MBCD-dependent cholesterol depletion inhibited the intracellular CIP50-coronin-1 interaction, (ii) addition of exogenous soluble cholesterol reversed the inhibitory effect of MBCD, and (iii) cholesterol was also required for CIP50/LpdC binding to coronin-1 in a cell-free system. Of particular relevance to these findings, analysis of the primary sequence of Mtb LpdC revealed three putative cholesterol-binding domains (L/V-(X_{1-5})-Y-(X_{1-5})-K/R) (Li et al., 2001) (amino acid positions: 75-85, 273-280 and 293-298) (see boxes in supplementary material Fig. S2) suggesting that targeting of cholesterol-rich lipid rafts by pathogenic mycobacteria results in a stable, cholesterol-mediated interaction between LpdC and coronin-1 in nascent vacuoles. Interestingly, the amino acid position V75, K280 and

V293 in the cholesterol-binding motifs of Mtb and BCG LpdC are substituted in the *M. smegmatis* LpdC sequence with A75, A280 and I293, respectively (supplementary material Fig. S2). Therefore, it is likely that these amino acid substitutions play a role in the binding of cholesterol to LpdC or in its secretion. Such an assumption is supported by the finding that *M. smegmatis* transfected with vector expressing the Mtb *lpdC* gene, was able to secrete substantial amount of LpdC and behaved like BCG in terms of prolonged retention of coronin-1 on the phagosome. These observations also established a direct cause-and-effect relationship between abnormal retention of coronin-1 on the phagosome and the intracellular release of LpdC.

It was important to demonstrate the relevance of LpdC-mediated retention of coronin-1 on BCG vacuoles to the arrest of phagosome maturation. We reasoned that if prolonged retention of coronin-1 modulated phagosome biogenesis in infected cells, it would be observed to dissociate relatively rapidly from phagosomes under conditions where mycobacterial vacuoles normally fuse with lysosomes. Indeed, in macrophages incubated with IFNγ [a cytokine known to promote maturation of mycobacterial phagosomes and inhibition of intracellular replication (Schaible et al., 1998; Via et al., 1998)] BCG vacuoles intersected LysoTracker-containing lysosomes concomitant with the release of coronin-1 from the bacterial vacuole.

IFNγ-induced expression of the antimicrobial enzyme NOS2 has been considered largely responsible for restricting Mtb replication via NO generation (Chan et al., 1992; MacMicking et al., 1997). However, the finding that the macrophage drastically reshapes its transcriptome in response to IFNγ [over 1000 genes are transcriptionally activated after stimulation of macrophages with IFNγ (Ehrt et al., 2001)] strongly suggested the possibility of additional TB defense pathways elicited by this cytokine. In this respect, recent in vivo studies have revealed the existence of NOS2-independent effector mechanisms against TB, mediated at least in part by a novel, small GTPase, LRG-47 (MacMicking et al., 2003). Furthermore, it was demonstrated that LRG-47 is a critical regulator of autophagy-dependent disposal of mycobacterial vacuoles (Gutierrez et al., 2004). In keeping with these findings, the present study showed that the block in acidification of BCG vacuoles was largely overcome in cells transfected with LRG-47. Moreover, LRG-47 recruitment to phagosome membranes was coincident with both the dissociation of coronin-1 from LpdC and its release from BCG vacuoles. These findings provide an additional basis for understanding how IFNγ-induced LRG-47 expression participates in the regulation of phagosome maturation.

In summary, the results presented above demonstrate the direct involvement of BCG/Mtb LpdC in the prolonged retention of coronin-1 on the phagosome, thus promoting phagosome maturation arrest. More importantly, they show that under the influence of IFNγ, LRG-47 is recruited to the phagosome and mediates dislocation of coronin-1 leading ultimately to phagosome acidification and recruitment of lysosomal vacuoles and phagolysosome fusion. Future studies should address the mechanisms of LRG-47-induced dissociation of the LpdC-coronin-1 complex from the phagosomal membrane and confirm the contribution of cholesterol binding motifs in LpdC-coronin-1 interaction.

Materials and Methods

Reagents and chemicals

Endotoxin-free culture reagents were from StemCell Technologies (Vancouver, British Columbia, Canada). Protease inhibitor mixture, PMSF, trypsin-EDTA, methyl-beta-cyclodextrin (MβCD), ganglioside GM1 and glutathione-agarose beads were purchased from Sigma-Aldrich (St Louis, MO). Protein A-agarose beads were from Bio-Rad laboratories (Hercules, CA). [^{35}S]methionine (specific activity 1 mCi/mmole) was from PerkinElmer (Boston, MA) and murine rIFNγ was a generous gift of Genentech (South San Francisco, CA).

Antibodies

Mouse mAb (N-7) and rabbit polyclonal anti-coronin-1 were described previously (Oku et al., 2003). Polyclonal anti-LpdC antibodies were raised in New Zealand white rabbits immunized with KLH conjugated synthetic peptide (LPNEDADVSKEIEKQ) corresponding to LpdC amino acids 207-220. Specific anti-LpdC antibodies were immunopurified using an immobilized peptide column prepared using the Sulfolink® kit (Pierce Biotechnology, Rockford, IL). Rabbit polyclonal anti-LRG-47 antibody was provided by Gregory Taylor (Duke University Medical Center, Durham, NC) and rabbit anti-coronin-1 was a gift from Jean Pieters (Basel Institute for Immunology, Switzerland). All secondary antibodies were purchased from Caltag Laboratories (Burlingame, CA) except Alexa Fluor 647-conjugated goat anti-rabbit IgG which was purchased from Invitrogen Canada Inc (Burlington, Ontario, Canada).

Cell culture

The murine macrophagic cell line J774A.1 (American Type Culture Collection, Manassas, VA), was cultured in DMEM supplemented with 2 mM L-glutamine, non-essential amino acids and 10% FCS (HyClone, Logan, UT). Prior to infection with mycobacteria, cells were seeded at a density of 10^5/cm^2 on coverslips or in 10 cm diameter culture dishes (Corning Inc., Corning, NY) and allowed to adhere at 37°C in a humidified atmosphere of 5% CO_2.

Mycobacterial strains

M. bovis BCG (Pasteur 1173P2) and *M. smegmatis* (mc^{2}155) strains expressing GFP (Cowley and Av-Gay, 2001) were used in this study along with Mtb H_{37}Rv. BCG expressing *Ebfp*, a gene encoding a blue fluorescent protein (BFP-BCG) was prepared using the plasmid construct pMN431 as described previously (Kaps et al., 2001). pMN431 was a gift from Michael Niederweis, University of Alabama, AL). Mycobacteria were grown in Middlebrook 7H9 broth (Difco, Franklin Lakes, NJ) supplemented with 10% (v/v) OADC (oleic acid, albumin and dextrose solution; Difco) and 0.05% (v/v) Tween 80 (Sigma-Aldrich) at 37°C on a shaker. Bacteria were harvested by centrifugation and pellets were suspended in complete medium plus 10% glycerol. Mycobacterial cultures were stored in aliquots (~5×10^8/vial) at –70°C. To generate radioactive culture filtrate proteins (^{35}S-CFPs), mycobacteria grown in 7H9-OADC were pelleted and cultured (for 16-18 hours for BCG and 3-4 hours for *M. smegmatis*) at 37°C in modified Dubos medium (0.1% KH_2PO_4, 0.05% Na_2HPO_4, 0.12% sodium citrate, 0.06% $MgSO_4$, 2% asparagine and 0.1% Tween 80) supplemented with 100 μCi/ml of [^{35}S]methionine. The culture supernatants were cleared from bacteria by high speed centrifugation and filtration through 0.22-μm-pore size filters. Bacteria were killed by 2 hours incubation at 37°C in the presence of 50 μg/ml gentamycin.

Recombinant *M. smegmatis* expressing Mtb LpdC

Recombinant *M. smegmatis* expressing GFP and Mtb LpdC (rMsmeg) was generated as follow: primers lpd-1: CCCAAAGGATCCGTGACCCACTATGACGTCGT (with a 5′ *Bam*HI adaptor) and lpd-2: CCCTTTGATATCTCAGAAAATTGATCATGTGG (with a 5′ *Eco*RV adaptor) were used to amplify the entire open reading frame (ORF) of *lpdC* from Mtb genomic DNA. The resulting amplicon was then cloned between the *Bam*HI and *Eco*RV sites of *E. coli/Mycobacterium* shuttle vector pSC301 (Kaps et al., 2001) to obtain the recombinant plasmid pSC-lpdC in which *lpdC* was placed under the control of the mycobacterial superoxide dismutase promoter in transcriptional fusion with the promoterless gfp cassette. The recombinant plasmid pSC-lpdC was then used to transform *M. smegmatis* mc2 strain. Transformants were selected on standard 7H10 agar medium in the presence of 50 μg/ml hygromycin.

Intracellular staining

Intracellular staining was performed on fixed and permeabilized cells as described previously (Hmama et al., 1999; Sendide et al., 2005a). Coverslips were mounted in FluorSaveTM (Calbiochem-Novabiochem Corp., La Jolla CA) to minimize photobleaching. Then slides were examined by digital confocal microscopy using an Axioplan II epifluorescence microscope (Carl Zeiss Inc., Thornwood, NY) equipped with 63×/1.4 Plan-Apochromat objective (Carl Zeiss Inc). Images were recorded using a CCD digital camera (Retiga EX, QImaging, Burnaby, BC, Canada) coupled to the Northern Eclipse software (Empix Imaging, Inc., Mississauga, ON, Canada).

Expression and purification of recombinant coronin-1

Recombinant coronin-1 protein was produced in *E. coli* JM109 as fusion proteins with GST as described previously (Oku et al., 2003).

Pull-down assays

Radiolabeled mycobacteria were extensively washed with PBS-0.1% Tween 80 and used to infect J774 macrophages. Cells were then lysed in extraction buffer (25 mM Tris-HCl pH, 7.5, 1 mM EDTA, 1 mM EGTA, 100 mM NaCl, 1% Triton X-100, 0.5% NP-40, 0.2 mM PMSF and protease inhibitor cocktail) for 20 minutes at 4°C and debris was removed by high speed centrifugation. Five hundred μg of soluble proteins were mixed with 5 μg of anti-coronin-1 mAb N-7 or irrelevant normal mouse IgG and incubated for 1 hour at 4°C. Alternatively, soluble proteins from non-infected J774 cells were mixed with 100 μl of ^{35}S-CFPs and incubated for 2 hours at 4°C prior to the addition of N-7 mAb. Protein A-agarose beads were added to the mixtures and the samples were incubated for an additional 30 minutes at 4°C. In other experiments, 50 μg of GST-fusion protein immobilized on agarose beads, were mixed with 100 μl of ^{35}S-CFPs and incubated for 2 hours at 4°C. Agarose beads were washed extensively and protein complexes were solubilized in 1× Laemmeli buffer and submitted to SDS-PAGE and X-ray radiography. To ensure that coronin-1 levels were equivalent at the end of the immunoprecipitation, 10% from each treatment sample was collected during the last wash in a separate tube and analyzed by SDS-PAGE and immunoblotting with polyclonal rabbit anti-coronin-1 IgG.

Immunoelectron microscopy

Immunogold staining was conducted at the EM Facility of iCAPTURE Centre (Saint Paul Hospital, Vancouver, BC, Canada). In brief, BCG-infected macrophages were fixed with 2% paraformaldehyde + 0.5% glutaraldehyde and embedded in 4% low melting point agarose. Immuno-gold labeling was performed on 55 nm sections obtained with a Leica UC6 Ultracut microtome (Leica Microsystems Inc, Bannockburn, IL) and the grids were examined with a Tecnai 12 electron microscope (FEI Company, Hillsboro, OR).

Two dimensional gel electrophoresis

Pulled-down proteins were solubilized in the rehydration solution (8 M urea, 2% CHAPS, 2% IPG buffer, 20 mM DTT and 0.002% Bromophenol Blue). Proteins were first separated according to their isoelectric point (pI) in the immobilin Dry-Strip using IPGphor isoelectric focusing system (Amersham Biosciences, Piscataway, NJ). After equilibration, proteins separated on the strips were layered on 10% SDS-PAGE gels and subjected to electrophoresis. Gels were then dried and exposed to X-ray films.

Nano-capillary HPLC ion trap mass spectrometry (LC-MS/MS)

To identify mycobacterial coronin-1 interacting protein, culture filtrate proteins (CFPs) were concentrated ~100 fold and incubated with GST-coronin-1 cross-linked to glutathione-agarose bead for 2 hours at 4°C. The material eluted from the beads was then separated by two-dimensional (2D) gel analysis and subjected to silver staining. The spot of interest was analyzed with LC-MS/MS of in-gel tryptic digests at the Uvic Proteomics Centre Facilities (University of Victoria, Victoria, BC Canada).

Transient transfections with LRG-47

pF25/LRG-47 plasmid carrying GFP-LRG-47 fusion protein (Gutierrez et al., 2004) (kindly provided by Gregory Taylor) and pEGFP-2C plasmid carrying GFP alone (Clontech, San Jose, CA) were prepared using Endofree plasmid maxiprep kit (Sigma-Aldrich). J774 cells adhering to 12-mm-diameter tissue culture-treated coverslips (Fisher Scientific, Nepean, ON, Canada) or 60-mm culture dishes were transfected with the GFP constructs using the Lipofectamine 2000 reagent (Invitrogen) as described previously (Sendide et al., 2005b).

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