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Role of Mycobacteria Effectors in Phagosome Maturation Blockage and New Drug Targets Discovery

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ABSTRACT

Tuberculosis remains a serious global health threat with nearly 10 million new cases and 1.7 million deaths every year. The emergence of multi-drug resistant (MDR) and extensively drug resistant (XDR) strains of *Mycobacterium tuberculosis* (Mtb) further complicates this problem. It is pressing to find new ways to combat Mtb. The success of Mtb is largely attributed to its ability to persist within macrophages by arresting phagosomal maturation. The bacterial proteins and lipids play important roles in this inhibition which involves several aspects of phagosomal maturation, including both fusion and fission events and recruitment of V-ATPases allowing acidification. Understanding the interaction between the pathogen and host macrophage is essential to eradicate or control tuberculosis. This review focuses on the mechanism of phagolysosome formation, the pivotal event for the fates of infection participants and abundance of novel drug targets. J. Cell. Biochem. 112: 2688–2693, 2011.

KEY WORDS: MYCOBACTERIUM TUBERCULOSIS; PHAGOLYSOSOME; SECRETORY EFFECTOR; DRUG TARGET

Mycobacterium tuberculosis (Mtb), the causative agent of tuberculosis, is usually inhaled via the droplet nuclei containing the pathogen then are uptaken by alveolar macrophages. Despite the multitude of immune defense mechanisms a host can deploy against Mtb, the bacteria can continue to persist owing to its subtle tactics. The outcome of the infection largely depends on the interaction between the pathogen and host, especially that within the macrophage. One of the most usual upshot of this interplay, also the most difficult scenario to handle, is the bacteria finally persist within host cells in a yet obscure state, most probably dormant non- or slowly-replicating state. Many factors contribute to this end and in-depth understanding of these factors, both from the pathogen aspect and host, will facilitate our effective measures discovery.

PHAGOSOME MATURATION

Phagocytosis of Mtb by macrophages initiates the formation of the phagosome, resulting from a series of membrane invagination, budding, and fusion events [Pieters, 2008]. The phagosome undergoes sequential fusion with early endosomes, late endosomes, and lysosomes [Desjardins et al., 1994]. Then different stages of maturation—early, intermediate and late phagosomes—culminate with the formation of phagolysosomes. During the course of maturation, phagosome becomes a highly acidic, oxidative, and degradative milieu, which is a full arsenal of antimicrobial features.

The early phagosome rapidly obtains many of the characteristics of early endosome, and is capable of fusion with sorting and recycling endosome, but not lysosome [Desjardins et al., 1997]. Its lumen has a near-neutral pH of around 6.3 and poor in hydrolytic activity [Mukherjee et al., 1997]. The Rho-GTPase Rab5A and its effector early endosome antigen 1 (EEA1) are two characteristic markers of this organelle, and are known to mediate the traffic between early endosome and phagosome [Flannagan et al., 2009]. Rab5A also interacts with other multiple effectors, including the p150–hvPS34 complex and soluble NSF-attachment protein receptor proteins (SNARE proteins). The Ser and Thr kinase p150 assists the recruitment of hVPS34, a class III phosphatidylinositol-3-kinase (PI3K) that generates phosphatidylinositol-3-phosphate (PI3P) on the early phagosomal membrane [Vieira et al., 2001]. PIP3 is believed to present a docking site for several effectors involved in the fusion of phagosomes and lysosomes, such as the EEA1 and the hepatocyte growth factor-regulated tyrosine kinase

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substrate (Hrs) [Pieters, 2008]. Furthermore, EEA1 interacts with syntaxin 13, a SNARE protein required for membrane fusion [McBride et al., 1999]. Thus, both Rab5A and EEA1 are necessary for phagosomal maturation to proceed.

The intermediate phagosome is a midway point between early and late phagosome, with retained Rab5 but loss of EEA1 [Flannagan et al., 2009]. The late phagosome, in turn, has undergone a drop in pH caused by the pumping of H^+ into the lumen by the acquisition of additional V-ATPases, resulting in a pH of about 5.5 [Desjardins et al., 1994]. The late phagosomal membrane has acquired the small GTPase Rab7A, which is a characteristic marker of this organelle [Progida et al., 2010]. Additionally, the late phagosome also enriches lysosomal-associated membrane proteins, (LAMP)-1, LAMP-2, LAMP-3/CD63, which are either imported from the Golgi complex or from late endosomes, in a mannose 6-phosphate-dependent or independent manner [Flannagan et al., 2009]. The late phagosome is incapable of fusion with early endosome. Irrespective of how it is acquired, Rab7 recruits several effectors to the vacuolar membrane. One such effector, Rab-interacting lysosomal protein (RILP) associates with Rab7, is thought to act as a bridge that tethers phagosomes to dynein-dynactin, a microtubule-associated motor complex [Harrison et al., 2003]. Strikingly, the motors can promote the extension of phagosomal tubules toward late endocytic compartments. The VpsC–homotypic protein sorting (HOPS) complex, which is required for Rab5–Rab7 conversion, probably serves a similar function in phagosome maturation [Rink et al., 2005]. The HOPS complex promotes vesicle docking and fusion with target membranes by modulating the activity of SNARE proteins [Chao et al., 2010]. VPS33B, a part of the HOPS complex, can bind to t-SNAREs and specifically associate with GTPases [Yang et al., 2000]. Thereby, it is possible that VPS33B is a key regulator of membrane docking and fusion. Inactivation of this protein results in the arrest of phago–lysosome fusion [Bach et al., 2008]. These results demonstrate that both Rab7-RILP and Rab7-HOPS are the mediators of late phagosome maturation. But PI3K antagonists block phagosome maturation despite the constitutive expression of Rab7 [Vieira et al., 2003], suggesting that a parallel, inositide-dependent event is also essential.

The maturation process culminates with the formation of the phagolysosome, the ultimate microbicidal organelle with active hydrolases and other microbicidal substances, a membrane protein composition similar to that of lysosomes, and an acidic environment with a pH value as low as 4.5 due to the vast number of V-ATPases in the membrane [Flannagan et al., 2009]. The phagolysosome can be distinguished from the late phagosome by their lysobisphosphatidic acid-, mannose 6-phosphate receptor-, and PI3P-poor internal membranes [Griffiths et al., 1988; Kobayashi et al., 1998; Gillooly et al., 2000], and by their elevated content of mature cathepsin.

EFFECTORS THAT INHIBIT PHAGOSOME MATURATION

There are several ways in which Mtb modulates the macrophage defences to promote its survival, and the inhibition of phagosomal maturation is the best characterized mechanism. This inhibition involves several aspects of phagosomal maturation, including both fusion and fission events and recruitment of V-ATPases allowing acidification. Here, it is important to realize that both proteins as well as (glyco)lipids play important roles in this inhibition[Haucke and Di Paolo, 2007], as illustrated in Figure 1 and Table I.

LAM

Mycobacterial lipoarabinomannan (LAM) plays an important role in the retention of phagosome on the early phage. LAM, a major lipoglycan of the *M. tuberculosis* cell wall, is shed by the bacterium upon entry into the cell and can thereafter be found throughout macrophage membranes [Shabaana et al., 2005]. The mannose-caps, only isolated from pathogenic mycobacteria, mediated the binding of LAM with host cell mannose receptors (MR). This interaction is important for the capability of purified LAM to arrest phagolysosome fusion of LAM-coated latex beads [Kang et al., 2005]. However, one study performed with live bacteria contradicts this paradigm [Appelmelk et al., 2008]. Regardless of how it is acquired, ManLAM can physically obstruct membrane fusion by inducing a dramatic reorganization of raft composition [Hayakawa et al., 2007].

Furthermore, there are several ways in which ManLAM inhibits the recruitment of EEA1(Fig. 1). Firstly, the inhibition is accomplished by interrupting a Ca^{2+} surge in the cytoplasm which is necessary for calmodulin- and Ca^{2+}/calmodulin-dependent kinase II (CaMKII)-dependent delivery of EEA1 [Malik et al., 2003]. Prevention of the Ca^{2+} surge is thought to be preceded by a blockage

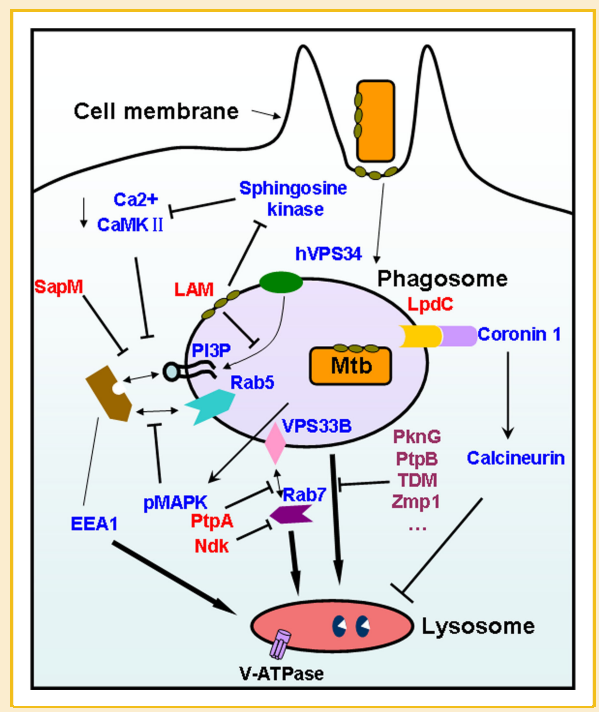

Fig. 1. Modulation model of host–pathogen interactions of the phagosome maturation. *M. tuberculosis* effectors are depicted in red, whereas host factors are blue. Brown represents the Mtb effectors that have not identified host targets yet. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

TABLE I. Effectors That Contribute to The Survival of Obligatory Intracellular Bacterial Pathogens

Effector	Cellular target	Effector function	Refs.
LAM	Unknown	Blocks cytosolic Ca^{2+} fluxes, suppressing hVPS34 activation	Malik et al. [2003]; Vergne et al. [2003a]
SapM	PI3P	Hydrolyzes PI3P, inhibiting phagosome-late endosome fusion	Vergne et al. [2005]
PtpA	VPS33B	Dephosphorylates VPS33B, arresting phagolysosome fusion	Bach et al. [2008]
LpdC	Coronin-1	Retains coronin-1 on the phagosomal membrane, arresting phagosome maturation	Deghmane et al. [2007]
Zmp1	Inflammasome	Prevents inflammasome and, therefore, IL-1β activation	Master et al. [2008]
Ndk	Rab5 and Rab7	Inactivation of both Rab5 and Rab7, thereby inhibiting their respective effectors recruitment	Sun et al. [2010]
PknG	Unknown	Acts by phosphorylating a host molecule, thereby preventing the activity of this host factor in carrying out phagosome-lysosome fusion	Pieters [2008]
PtpB	Unknown	Might be capable of disrupting host phosphoinositide metabolism and its associated signaling pathways	Beresford et al. [2007]
TDM	Unknown	Mediates intracellular trafficking events, as well as influence macrophage production of pro-inflammatory molecules	Indrigo et al. [2003]

of a macrophage sphingosine kinase [Malik et al., 2003]. A second mechanism is actively preventing PI3P accumulation. This is thought to occur through ManLAM-mediated inhibition of the PI3 kinase hVPS34 as well as through SapM-mediated dephosphorylation of PI3P [Vergne et al., 2003a]. In summary, the events described above lead to reduced Rab5 activity and thus inhibit the recruitment of EEA1, resulting in block the fusion of phagosome and late endosome or lysosome and can not gain the V-ATPases.

LpdC

Coronin 1 (also known as TACO) is an important host factor that specifically prevents the lysosomal delivery and death of mycobacteria inside macrophages by regulating calcium-dependent signaling processes [Ferrari et al., 1999; Gatfield et al., 2005]. Upon viable mycobacterial entry into phagosomes, coronin 1 is recruited to the mycobacterial phagosome and did not bind to any other subcellular organelle [Ferrari et al., 1999]. Consistent with an important role for coronin 1 in inhibiting phagosome maturation, BCG is delivered to a lysosomal lumen in macrophages lacking coronin-1 [Jayachandran et al., 2007].

Deghmane et al. [2007] identified mycobacterial lipoamide dehydrogenase C (LpdC) as a coronin-1 binding protein. LpdC forms an integral component of peroxynitrite reductase/peroxidase, which was recently reported to be crucial for mycobacterial antioxidant defenses [Rhee et al., 2005]. The authors demonstrated that LpdC actively retains coronin-1 on the phagosomal membrane, and this interaction is dependent on cholesterol. LRG-47, a critical regulator of autophagy-dependent disposal of mycobacterial vacuoles [Gutierrez et al., 2004], can largely overcome the blockage in acidification of BCG vacuoles. The exactly mechanism is still unclear, but LRG-47 recruitment to phagosome membranes was coincident with both the dissociation of coronin-1 from LpdC and its release from BCG vacuoles. Interestingly, a lipoamide dehydrogenase, which is involved in intracellular bacterial metabolism, would play an extracellular role in phagosome maturation, and it is unclear whether these functions are related.

PknG

The secreted serine/threonine protein kinase G (PknG) is the only soluble kinase maintained in the genome of all pathogenic mycobacteria, and is only required for the survival of *M. tuberculosis* in vivo, but not in vitro [Nguyen et al., 2005]. Mtb's PknG was thought to act as an important mediator of phagosome maturation inhibition. One piece of evidence is that inactivation of PknG by gene disruption or chemical inhibition resulted in the maturation of the Mtb-phagosome and intracellular killing of the bacteria [Briken, 2008]. The mechanism of cytosolic translocation of PknG and the precise action of PknG on host trafficking machinery is not known. However, as several factors that are involved in the regulation of intracellular transport reactions require phosphorylation, PknG is proposed to function through the phosphorylation of an unknown host protein, thereby preventing the activity of this host factor in carrying out phago-lysosome fusion [Pieters, 2008].

Interestingly, the *M. smegmatis*-PknG expressing BCG and BCG-PknG expressing *M. smegmatis*, but not wild type *M. smegmatis*, are able to prevent translocation of the bacteria to lysosomes [Walburger et al., 2004]. Furthermore, the PknG of WT *M. smegmatis* shares 78% identity and 87% similarity with Mtb- and BCG-PknG. Why the PknG is invalid in the non-pathogenic mycobacteria? Houben et al. [2009] found that PknG is transcribed but not translated in *M. smegmatis* due to regulatory elements in the upstream region of the *pknG* operon. However, an earlier publication shows the opposite result that PknG protein was successfully isolated from *M. smegmatis* cell extracts using the PknG substrate GarA as bait [O'Hare et al., 2008]. Therefore, Joseph et al. suggested, based on these observations mentioned above, that the reason for lysosomal transfer observed in the BCG Δ*pknG* mutant is ascribed to physiological changes within mycobacteria but did not affect host-signaling pathways [Chao et al., 2010].

PtpA

PtpA, a low-molecular-weight tyrosine phosphatase, is supposed to modulate host tyrosine phosphorylated protein(s) due to the lack of tyrosine kinases in the Mtb genome [Bach et al., 2008]. Initial studies demonstrated that purified recombinant PtpA is specific for phosphotyrosine-containing substrates and its expression is upregulated during entry into human macrophages [Cowley et al., 2002]. Like PknG, PtpA is not required for the survival of in vitro growth, but is shown to be essential for successful Mtb infection of human macrophages using a *ptpA* gene deletion mutant [Bach et al., 2008]. The experimental data presented above greatly

spurred interest in the detection of the substrates of PtpA and its physiological function in host macrophages during infection. VPS33B, a host cytosolic protein, was identified as a PtpA substrate by a "substrate trapping" assay [Bach et al., 2008]. As mentioned above, VPS33B is part of the HOPS complex; it binds to t-SNAREs and can specifically associate with GTPases. Therefore, it is likely that the mechanism of PtpA inhibits phago-lysosome fusion and dephosphorylation of VPS33B leading to a block of the macrophage's membrane docking machinery by obstructing the association of VPS33B with the small GTPases essential for proper membrane fusion [Chao et al., 2010]. However, PtpA is not required for Mtb growth in a mouse infection model, suggesting that mice model cannot completely mimic the niche of human macrophage.

PtpB

The protein phosphatase PtpB is an essential secreted virulence factor in *M. tuberculosis*. A combination of bioinformatics analysis, enzyme kinetics, and substrate-specificity characterization revealed that MptpB can dephosphorylate phosphotyrosine, phosphoserine/threonine, and phosphoinositides substrates, thereby exhibiting triple-specificity [Koul et al., 2000; Vergne et al., 2005; Beresford et al., 2007]. Crystallographic analysis revealed that PtpB possesses the distinct features of dual phosphotyrosine binding sites. Based upon these results, the authors suggested a potential role of PtpB in host phosphoinositide metabolism and its associated signaling pathways, which are known to have a key role in phagosome maturation. An earlier publication shows that disruption of the *ptpB* gene impairs the ability of the mutant *M. tuberculosis* to survive in activated murine macrophages and resulted in 70-fold reduction of bacillary loads in the spleens of infected guinea pigs [Singh et al., 2003]. Importantly, an inhibitor of PtpB (isoxazole) added to macrophages during infection led to severely impaired intracellular mycobacterial growth [Beresford et al., 2009], further demonstrating an essential role of PtpB in the intracellular survival of Mtb. Yet, further investigations are needed to identify the role of PtpB in the pathogenesis of TB, especially elucidation of its cognate substrates.

OTHER EFFECTORS

Indrigo et al. (2003) found the cord factor trehalose 6,6′-dimycolate (TDM), one important cell wall component, is a determinant for successful infection and survival of Mtb within macrophages due to mediate trafficking events. Both latex beads model [Indrigo et al., 2003; Axelrod et al., 2008] and Δ*fbpA* mutants [Katti et al., 2008], which are defective in transferring mycolic acids to trehalose to generate TDM, are exhibiting altered phagosome maturation. The mechanism of action and the cognate substrate of TDM is unclear. Mycobacterial nucleoside diphosphate kinase (Ndk) is a putative virulence factor that inhibits phagosome maturation and its recombinant form exhibits GTPase activating protein (GAP) activity towards Rab5 and Rab7 [Sun et al., 2010]. A model of latex bead phagosomes and the Ndk knocked-down BCG strains demonstrated that Ndk inhibits phagosome maturation and promotes survival of mycobacteria within the macrophage [Sun et al., 2010]. An Mtb gene, *zmp1*, encoding a putative Zn^{2+} metalloprotease, could therefore inhibit phagosome maturation by preventing IL-1β activation [Master et al., 2008]. Moreover, p38MAPK activation by some effectors of the Mtb reduced Rab5 activity and thus reduced EEA1 on phagosomes [Fratti et al., 2003]. Mtb and *M. marinum* mutants defective in the Esx-1 secretion system also show defects in arresting phagosome maturation [MacGurn and Cox, 2007]. In *M. tuberculosis*, known substrates of the Esx-1 system (ESAT-6, CFP-10, and EspA) appeared dispensable [MacGurn and Cox, 2007], suggesting the existence of other additional unidentified effectors.

INSPIRATION FOR NEW DRUG TARGETS

Tuberculosis, the only disease ever declared a global emergency by the WHO, accounts for nearly two million deaths per year. The need for shortened treatment regimen as well as the emergence of multi-drug resistant (MDR) and extensively drug resistant (XDR) MTB strains, possibly a dire result of prolonged therapeutic exposure to drugs, necessitate discovery of new drugs. Mtb proteins/lipids involved in phagosome maturation blockage hold great promise for better treatment, as this would enable the macrophage to overcome infection as exemplified by the PknG inhibitors.

It is well known that most currently available effective tuberculosis drugs function by interfering directly with mycobacterial physiology. However, blocking the activity of secreted effectors such as PknG may allow the macrophage to carry out its innate antimicrobial activity, redirecting intracellular residing mycobacteria from phagosomes to lysosomes, thereby inducing and accelerating their destruction. Because the secreted effector is pathogen-specific, their inhibitors should not endanger the normal human microflora and cause a lesser side effect. An additional advantage of targeting secreted effectors is that their inhibitors do not need to overcome the extremely impermeable mycobacterial cell wall.

The requirement of the PknG kinase for mycobacterial survival inside macrophages has spurred the search for specific kinase inhibitors. Using combined screening and medicinal chemistry strategies, Walburger et al. [2004] identified AX20017, a tetrahydrobenzothiophene compound specific for PknG without any obvious adverse effect on macrophages or mycobacterial viability outside host cells. Indeed, a highly selective inhibitor for PknG has been recently identified [Scherr et al., 2007]. ESX-1 is absolutely required for pathogenesis of virulent mycobacteria and, therefore, the development of drugs against the core proteins of this secretion system are likely to disrupt the interaction between virulent Mtb with host cells at multiple levels and thus increase the efficacy of these drugs.

In summary, targeting these pathogenic secreted effectors instead of the general housekeeping proteins and lipids, might have the advantage of identifying drugs that specifically target pathogenic bacteria without damage to normal microflora. Furthermore, these drugs can surmount the impervious mycobacterial cell wall, thereby increasing the drug's efficacy indirectly.

CONCLUDING REMARKS

Upon entry into macrophage, viable MTB can regulate the biogenesis and maturation of endosomes, resulting in the phago-

somes incapable of obtaining the features of lysosomes. The MTB can snatch the iron and other nutrients essential for growth and proliferation; meanwhile, it can clear the calcium ions, hydrogen ions, and H-ATPase which are pernicious to their survival. Finally, the MTB inhibits the fusion of phago-lysosome, thereby establishing a relative safe intracellular niche. MTB effectors that inhibit the fusion of phago-lysosome have been identified. These factors might synergize in one way or other to gain the maximum advantage. For example, SapM is postulated to function along with ManLAM to obviate the recruitment of PI3P for the bacterial phagosome, while PtpA and Ndk may act in concert to disrupt Rab7 recruitment and activation. Advances in technology and in-depth study will further elucidate the sophisticated stratagem employed by this clever pathogen and facilitate our countermeasures design.

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