Rab32 restriction of intracellular bacterial pathogens

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ABSTRACT

Our immune system is engaged in a continuous battle against invading pathogens, many of which have evolved to survive in intracellular niches of mammalian hosts. A variety of cellular processes are involved in preventing bacterial invasion or in killing bacteria that successfully invade host cells. Recently, the Rab GTPase Rab32 emerged as critical regulator of a host defense pathway that can eliminate bacterial pathogens. *Salmonella enterica* is an intracellular bacterium and a major cause of infections and deaths in humans. Rab32 and its guanine nucleotide exchange factor BLOC-3 are essential to prevent the growth of the human-restricted *Salmonella enterica* serovar Typhi (S. Typhi) in mice, a non-susceptible host. The importance of the Rab32/BLOC-3 pathway has been recently indicated by the finding that broad-host *Salmonella enterica* serovars deliver 2 bacterial effectors to neutralize this pathway and infect mice. Rab32 has also been shown to control infection by *Listeria monocytogenes*, another medically relevant intracellular pathogen. In addition, genetic evidence indicate a possible role of Rab32 in controlling leprosy, a disease caused by *Mycobacterium leprae* in humans, suggesting that a Rab32-dependent pathway can also act as a host defense pathway in humans. The Rab32 role in bacterial pathogen restriction is discussed here and compared to the function of this GTPase in other cellular processes.

Introduction

Many cellular processes are involved in preventing bacterial pathogens from replicating within mammalian hosts (reviewed in refs. 1, 2). Phagocytic cells (i.e., macrophages and dendritic cells) are the cells of the immune system devoted to internalizing and destroying bacterial invaders. Phagocytic processes are well studied, as well as many of the processes that lead to the activation of antimicrobial mechanisms and bacterial removal. Reactive oxygen species, nitric oxide, antimicrobial peptides and toxic concentrations of heavy metals are part of the molecular arsenal used by phagocytic cells to kill bacterial pathogens. However, successful bacterial pathogens evolved to neutralize this antimicrobial attack and survive within phagocytic cells. Investigating the bacterial molecules involved in this host-pathogen battle has recently led to the discovery of a novel host defense pathway, where a central role is played by the Rab GTPase Rab32. This pathway protects some mammalian species, e.g. mice, from the attack of the human-adapted pathogen *Salmonella enterica* serovar Typhi (S. Typhi). However, it does not protect mice from broad-host *Salmonella enterica* (Salmonella) serovars, as these serovars have evolved potent bacterial effectors to neutralize this pathway. The Rab32-dependent pathway might be a broad host-defense pathway against intracellular pathogens. In fact, genetic evidence suggests a role for Rab32 in preventing *Mycobacterium leprae* infections. In addition, Rab32 and its close homolog Rab38 have also been reported to control *Listeria monocytogenes*. In this mini-review we discuss the findings that led to the identification of the Rab32 host defense pathway in the context of *Salmonella* infections, evidence of its involvement in controlling other intracellular pathogens and the role of Rab32 in other cellular trafficking processes.

Salmonella: Establishing different diseases in different hosts

*Salmonella* is a facultative intracellular pathogen that is well adapted to live in mammalian hosts. There are more than 2 thousand serovars belonging to this species and they constitute a main cause of infectious diseases. *S. Typhi* and the less common *Salmonella enterica* serovar Paratyphi A (S. Paratyphi) are responsible for more than 26 million cases of typhoid fever, a life-threatening disease that kills hundreds of thousands of people each year, mainly in the developing world. In contrast, all...
the other Salmonella serovars cause in humans a milder and local infection, i.e. a gastroenteritis known as salmonellosis. Remarkably, while S. Typhi and S. Paratyphi are host-restricted serovars that can only infect humans, the majority of Salmonella serovars can infect a broad-range of animal hosts and cause, in many of these hosts, a systemic disease that is often fatal. For example, the well-known Salmonella serovar Typhimurium is responsible for common outbreaks of gastroenteritis in humans but can infect systemically cattle, sheep and mice, with the last being a common laboratory model of Salmonella systemic infection.

Salmonella colonizes the host by initially invading the epithelial cells lining the intestinal tract. This invasion is an active process induced by Salmonella via the delivery of effectors through a specialized secretion system, called type III secretion system. These effectors act coordinately to induce macropinocytosis and the formation of a compartment known as the Salmonella-containing vacuole, where this pathogen thrives and multiplies. Some of the type III secretion effectors also participate in inducing an inflammatory response that confers Salmonella an advantage over commensal intestinal bacteria. Once Salmonella has multiplied in the intestinal epithelial cells, the bacterium can get access to the underlying connective tissue or lamina propria. The lamina propria is rich in macrophages and other immune cells that mediate the initial immune response to pathogens. In this tissue an important battle is played at the level of the macrophages and possibly other phagocytic cells. These cells can eliminate bacteria, including many pathogens. However, if the bacteria succeed in overcoming host-cell defenses that act to restrict bacterial growth, the host will not be able to mount an immune response to the bacteria and the infection will spread systemically. Bacteria will survive and multiply in phagocytic cells, with the result that the same cells that should have restricted the infection actually become a vehicle to spread the infection to the bloodstream and peripheral organs. Depending on the specific host and the specific Salmonella serovar involved, either the bacterium will be efficiently killed in the intestinal lamina propria or a systemic infection will develop. The function of a second type III secretion system is required for a Salmonella broad-host serovar, S. Typhimurium, to establish a systemic infection in the mouse. This second type III secretion system is only expressed once the bacteria have established their intracellular niche, and is required for the bacteria to survive and replicate in macrophages. However, the exact mechanisms through which the effectors delivered by this system favor bacterial survival and the establishment of a systemic infection are only recently starting to emerge.

**Salmonella Typhi host-restriction: Critical role of a Rab32/BLOC-3-dependent trafficking pathway**

S. Typhi can only infect humans. Although it is responsible for a life-threatening disease that has been affecting humans for centuries, the molecular mechanisms at the basis of its human-adaptation and the ability to spread systemically are still mostly unknown. For many years, the entry and trafficking of the broad-host serovar S. Typhimurium within infected cells, particularly epithelial cells, have been investigated in detail. S. Typhimurium is first internalized in an EEA1 positive compartment, which then acquires Rab7 and endolysosomal markers, such as the glycoprotein LAMP-1. Only recently a major difference in the intracellular biology of S. Typhi and S. Typhimurium has been reported. S. Typhi recruits the GTPases Rab32, Rab38 and Rab29 (also known as Rab7L1) to its vacuole, but S. Typhimurium does not. These 3 proteins constitute a subfamily of Rab GTPases distinct from the primordial endocytic and exocytic Rab subfamilies. Both in epithelial cells and macrophages the majority of S. Typhi-containing vacuoles are decorated by Rab32, Rab38 and Rab29, while these Rab GTPases are never recruited to the S. Typhimurium-containing vacuole. What is the molecular basis for this striking difference? Why does the S. Typhimurium vacuole not acquire Rab32, Rab38 and Rab29? The reason resides in 2 bacterial effectors that target these GTPases. They are both expressed and delivered by S. Typhimurium, but not S. Typhi. The first to be identified was GtgE, a bacteriophage protein previously shown to be required for S. Typhimurium virulence in mice. GtgE is a cysteine protease that specifically cleaves these 3 related Rab GTPases. The second S. Typhimurium effector targeting Rab32 is SopD2, a protein that acts as a GTPase activating protein (GAP) for Rab32 and induces its dissociation from the vacuolar membrane.
conserved in the majority of broad-host Salmonella serovars and absent from S. Typhi and S. Paratyphi, the hypothesis was made that it confers Salmonella serovars the ability to infect a broad-range of hosts. This hypothesis was initially tested in primary murine macrophages, which are able to clear the human-restricted S. Typhi and prevent S. Typhi from infecting mice. When GtgE was introduced in the S. Typhi genome, macrophages were no longer able to kill S. Typhi. In addition, mice inoculated with a strain of S. Typhi expressing GtgE were systemically infected with this strain. This ability of GtgE to enhance S. Typhi survival in mouse macrophages and infectivity in mice was shown to be the result of its protease activity toward Rab32. In fact, murine macrophages depleted of Rab32 were found to be unable to clear S. Typhi. Rab38 and Rab29 are also substrates of GtgE, but siRNA-mediated depletion of these 2 Rabs has no effect on the ability of macrophages to kill S. Typhi. Rab38 is highly related to Rab32 and has been shown to work redundantly with Rab32 in other cellular processes. However, as Rab38 expression is limited to specific tissues, it is probably not expressed in primary murine macrophages.

Over the past decade Rab32 and Rab38 have emerged as critical molecules in the biogenesis of lysosomal related organelles (LROs), as discussed below. The Biogenesis of Lysosome-related Organelle Complexes (BLOC) 1, 2, and 3 are also fundamental players in the biogenesis and maturation of these organelles. In addition to Rab32, one of these complexes, BLOC-3, was shown to be required for S. Typhi killing in primary murine macrophages. Interestingly, at the same time BLOC-3 was reported to be the guanine nucleotide exchange factor for Rab32 and Rab38. All these observations indicated the existence of a Rab32 regulated trafficking event controlling the killing of S. Typhi in primary murine macrophages. Noteworthy, mice defective for BLOC-3 were also found to be more susceptible to S. Typhi infection. Indeed, while wild-type mice completely clear S. Typhi infection, S. Typhi can be recovered from tissues of BLOC-3-deficient mice 2 months after the initial inoculation. These results indicate that the Rab32/BLOC-3 trafficking pathway is central for S. Typhi-host restriction.

The Rab32/BLOC-3 trafficking pathway is neutralized by broad-host Salmonella serovars

Does the Rab32/BLOC-3 trafficking pathway only restrict S. Typhi infections? Interestingly, both BLOC-3-deficient mice and Rab32-deficient mice are significantly more susceptible to the broad-host Salmonella serovar S. Typhimurium. Surprisingly, removing GtgE from S. Typhimurium does not significantly impair the ability of S. Typhimurium to infect mice and does not restore Rab32 on the Salmonella-containing vacuole, suggesting that S. Typhimurium also inhibits the recruitment of Rab32 to the bacterial vacuole through a different effector. This S. Typhimurium effector was identified as SopD2, an effector that is not expressed by S. Typhi due to the fact that it is present as a pseudogene in the S. Typhi genome. SopD2 is a GTPase activating protein (GAP) that inactivates Rab32. As such, it prevents Rab32-dependent trafficking in S. Typhimurium-infected cells and confers this broad-host Salmonella serovar the ability to infect mice (Fig. 1). Remarkably,

Figure 1. Rab32/BLOC-3 dependent antimicrobial pathway and the mechanisms used by S. Typhimurium to neutralize it. After entry into a host cell, Salmonella resides in a compartment known as the Salmonella-containing vacuole. In contrast to S. Typhi (right panel), S. Typhimurium (left panel) delivers to the host 2 effector proteins, GtgE and SopD2. Both effectors block the Rab32/BLOC-3-dependent antimicrobial pathway by targeting Rab32. This pathway is envisioned to deliver an antimicrobial factor (small red circles) to the Salmonella-containing vacuole. GtgE is a protease that cleaves Rab32, whereas SopD2 acts as a GAP to inactivate Rab32 and promotes its dissociation from the vacuole. In addition, SopD2 interacts with Rab7 and inhibits its activity, which is required for the delivery of endosomal contents to lysosomes. EE, early endosome; LE, late endosome; Lys, lysosome.
deletions of both GtgE and SopD2 from the S. Typhimurium genome render S. Typhimurium unable to infect mice. SopD2 has also been recently shown to inhibit another trafficking process, the delivery of endocytic cargo to lysosomes. SopD2 acts as an inhibitor of the GDP-GTP exchange on Rab7 through direct binding of Rab7.38 The N-terminus of SopD2 is sufficient for Rab7 binding and inhibition of Rab7 function,38 while a C-terminal arginine (R315) is required for the SopD2 GAP activity on Rab32.10 These observations indicate that SopD2 has 2 different functions that can be assigned to different components of its structure.

Either the protease GtgE or the GAP SopD2 are able to prevent most of the Rab32 delivery to the vacuole and confer S. Typhimurium most of the ability to infect mice. Therefore, these effectors act in effect redundantly to block Rab32 trafficking and facilitate the establishment of a systemic infection in the mouse. Interestingly, a S. Typhimurium mutant defective for GtgE and SopD2, although unable to infect wild-type mice, can establish a systemic infection in mice deficient for the Rab32/BLOC-3-dependent pathway, indicating that GtgE and SopD2 are completely dispensable in the absence of this antimicrobial pathway.10 These recent results confirmed a central role for the Rab32/BLOC-3 trafficking pathway in host defense.

Rab32 and related GTPases in controlling other intracellular bacterial pathogens

Is the Rab32/BLOC-3-dependent host-defense pathway also able to restrict other intracellular pathogens? The genus Mycobacterium contains a number of species that cause serious diseases in humans, such as tuberculosis and leprosy. After internalization into host macrophages Mycobacteria reside in a compartment (phagosome) where they establish a suitable niche for survival.3,39 Mycobacterium leprae (M. leprae) is a human-adapted pathogen that causes a chronic infectious disease called leprosy.40,41 Most individuals exposed to M. leprae do not develop the disease, which may be explained, at least in part, by innate resistance provided by an individual’s genetic background. Several studies have demonstrated a link between host genetic factors and susceptibility to leprosy (for reviews see refs. 42, 43). Genome-wide association studies of leprosy have been critical to identify loci associated with susceptibility to M. leprae.11,12,44 Most of the identified risk loci encode factors involved in immune responses against infections. Remarkably, a polymorphism in the Rab GTPase Rab32 was also associated with increased susceptibility to M. leprae.11,12 There is a significant lack of knowledge on how eukaryotic cells deal with M. leprae infections at a molecular level. The challenge of cultivating M. leprae outside its host together with the limited availability of suitable animal models for the study of leprosy have limited research on the mechanisms at the basis of this disease. However, the emerging importance of Rab32 trafficking pathway in the cell defense response against bacterial pathogens strongly suggests that this Rab protein may play similar roles in controlling M. leprae infections (Fig. 2).

In contrast to M. leprae, the interaction of the related pathogen Mycobacterium tuberculosis (M. tuberculosis) with the host has been investigated at cellular level and several Rab GTPases have been implicated in the interaction. At an early stage, the M. tuberculosis phagosome...
lacks antimicrobial properties. The capacity to kill pathogens is acquired during a process of phagosome maturation, which includes phagosome acidification and acquisition of oxidative and hydrolytic enzymes. This process requires the interaction of the phagosome with components of the endocytic pathway, including Rab5 and Rab7. While Rab5 associates with early phagosomes, Rab7 characterizes late phagosomes and promotes their fusion with lysosomes. *M. tuberculosis* controls the recruitment of different Rab GTPases to the phagosome during the time of infection maintaining a niche with features of an early endocytic compartment. In addition to Rab5, the *M. tuberculosis* phagosome also recruits Rab14 and Rab22a, both contributing to the maintenance of phagosomes in an early stage of maturation. On the contrary, the recruitment of Rab GTPases related with latter steps of phagosomal maturation is prevented. Indeed, Rab7 and Rab20, which are involved in phagosome acidification, as well as Rab10, which is involved in early stages of maturation, are not observed on the *M. tuberculosis* phagosome. Therefore, a series of Rab GTPases regulated events avoid phagosome maturation and maintain an appropriate niche for *M. tuberculosis* survival. A possible role of the Rab32/BLOC-3 pathway in the control of *M. tuberculosis* survival is still unknown.

*Listeria monocytogenes* (*L. monocytogenes*) is an intracellular pathogen that causes listeriosis, one of the most lethal foodborne infections, with 20 to 30% of clinical infections resulting in death. The bacterium is internalized into phagocytic cells and non-phagocytic host cell through receptor-mediated recognition of specific bacterial proteins. Once internalized, *L. monocytogenes* actively escapes from the bacterial vacuole into the cytosol and then is transmitted to neighboring cells through actin-based movements induced by ActA-driven actin polymerization. *L. monocytogenes* killing in dendritic cells has been recently shown to be dependent on Rab32 and Rab38. The authors showed that the Rab32-positive compartment containing *L. monocytogenes* in dendritic cells is surrounded by a multilayered membrane. They also reported that Rab32 is recruited to the surface of bacteria that have escaped to the cytosol, suggesting that this Rab32-positive compartment containing *L. monocytogenes* is a compartment sequestering bacteria from the cytosol after their initial attempt of escape from the vacuole (Fig. 2). However, they also showed that the Rab32-dependent control of *L. monocytogenes* growth is not mediated by autophagic mechanisms. Further investigations will be required to understand the exact mechanism of Rab32-mediated pathogen killing. Valuable hints could derive from the analysis of Rab32 trafficking function in non-phagocytic cells.

**Rab32 subfamily of GTPases as regulators of membrane trafficking to lysosome-related organelles (LROs)**

Rab32 and its closely related isoform Rab38 have both been described as regulators of membrane trafficking to lysosome-related organelles (LROs). Rab38 expression is mostly confined to pigment cells. Rab32 is highly expressed in a variety of tissues including the liver, heart, spleen and testis, suggesting it performs a key role in these tissues. Expression levels are high in cells containing LROs. LROs are cell-type specific vesicles that share many similarities with lysosomes, yet perform a diverse range of functions. These include melanosomes, platelet granules, the MHC class II compartment and lamellar bodies. The observation that S. Typhi and *L. monocytogenes* recruit Rab32 to their vacuoles suggests that the S. Typhi- and the Listeria-containing vacuoles are also LROs. Many LROs have a critical function in immune defenses. Some of them, such as the lytic granules of T cells or the neutrophil granules, have a secretory function and they have also been called “secretory lysosomes.” Lytic granules contain molecules, such as perforin and granzymes, that, when released extracellularly, mediate target cell death. The neutrophil granules are filled with a variety of hydrolytic enzymes and antimicrobial peptides that are able to kill bacteria once secreted into the extracellular environment. Given the similarities between LROs and lysosomes and the fact that these 2 organelles exist within the same cell in many cell types, there must be mechanisms to distinguish between them and ensure the correct membrane proteins and luminal contents are appropriately trafficked to their target organelle.

Some genetic conditions result in deficiencies of LROs. Hermansky-Pudlack syndrome (HPS) is a genetic disorder that results in hypopigmentation, prolonged bleeding and pulmonary fibrosis, caused by defects in the biogenesis of melanosomes, platelets and lung fibroblast lamellar bodies, respectively. HPS is associated with mutations in BLOC-1, BLOC-2, BLOC-3 and AP-3. Rodent models of HPS display pigmentation defects (Swank et al., 1998). Interestingly, *chocolate* mice, which have a mutation in Rab38, display a mild pigmentation phenotype. This is in stark contrast to the severe hypopigmentation found when melanocytes from *chocolate* mice were depleted of Rab32 by siRNA treatment. Recently, the contribution of these Rabs in melanosome biogenesis has been explored in greater detail. Rab32 and Rab38 function within a trafficking pathway that transports melanin-producing enzymes to maturing melanosomes and interacts with components of ubiquitous trafficking machineries, such as BLOC-2, AP-1 and
Membrane-bound Rab32 and Rab38 were shown to physically interact with these proteins and this interaction was enhanced between the active GTP-bound forms of the Rabs and BLOC-2 and AP-3. This is consistent with an interaction between these proteins and the active membrane bound forms of Rab32 and Rab38.

Platelets are anucleated blood cells that play an essential role in homeostasis and host defense against pathogens. Platelets contain 2 types of LROs, α-granules (AGs) and dense granules (DGs), which are released at the site of vascular injury. It has been shown that DGs originate from late endocytic organelles (possible MVBs) in a model cell line and that the sorting signals that are required to send cargo to DGs are recognized by AP-3. These cells also contain conventional lysosomes suggesting mechanisms must exist to target molecules to AGs and DGs and away from other endocytic organelles. Both Rab32 and Rab38 colocalise with AP-3 and are enriched on immature DGs. Moreover, both Rab32 and Rab38 colocalise with Rab7, but not Rab5, reinforcing the hypothesis that DGs have a late endocytic origin. Both Rab32 and Rab38 are involved in the transport of cargo-containing vesicles to DGs but their exact role in vesicle tethering or fusion has not been separated. However, siRNA depletion of either Rab32 or Rab38 cannot be fully compensated by the remaining Rab GTPase suggesting they may fulfill separate functions in platelet granule maturation.

Rab32 appears to be a multifunctional protein, depending upon its cellular localization and the cell type. Rab32 has been shown to function as an A-kinase anchoring protein (AKAP) at both mitochondria and LROs. AKAPs act to retain other signaling enzymes at specific intracellular locations in order to coordinate protein phosphorylation events and direct them toward their substrates. Rab32 and Rab38 have been demonstrated to function within trafficking pathways for the delivery of proteins to both dense granules and melanosomes, in platelets and melanocytes, respectively. These specialized trafficking pathways employ unique trafficking machineries, including AP-3, BLOC-1, BLOC-2 and BLOC-3. This pathway is distinct from that of conventional lysosome biogenesis. As such, it would not be surprising to find that Rab32 (and Rab38) had a role in the biogenesis of other LROs, such as the lamellar bodies of lung type II epithelial cells. However, this remains to be seen.

Conclusions

Rab32 and its closely related homolog Rab38 control the delivery of specialized cargo to LROs. They are also recruited to the vacuoles containing bacterial pathogens, such as S. Typhi and L. monocytogenes, and they are essential to protect mammalian hosts, e.g., mice, from these pathogens. Rab32 and its GEF BLOC-3 are fundamental to restrict S. Typhi in mouse and are components of a powerful host defense pathway. A bacterial pathogen that can successfully infect mice, such as S. Typhimurium, has evolved 2 bacterial effectors to target and neutralize this pathway. The exact mechanism underlying the Rab32/BLOC-3-dependent clearance of bacterial pathogens is still unknown. However, based on the fact that Rab32 and the related Rab38 control the delivery of specialized cargo to LROs in melanocytes and platelets, it is very likely that this pathway delivers a cargo with antimicrobial properties to the S. Typhi- and the L. monocytogenes-containing vacuole. Future work clarifying the nature of this cargo will have a tremendous impact in the understanding of innate immune mechanisms protecting against bacterial pathogens.

Disclosure of potential conflicts of interest

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