

Bacterial Virulence Strategies That Utilize Rho GTPases

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Abstract The ability to modify central host cellular functions is a major advantage to many bacterial pathogens that use such strategies as part of their virulence mechanisms. Small GTPases, including Rho GTPases, make particularly attractive targets for pathogens because of their central roles in modulating cellular functions such as cytoskeletal control. Such modifications of these GTPases can include direct chemical modification of the GTPase or interfacing with some of the regulatory elements associated with GTPase control. Pathogens use these alterations in GTPase functions for a variety of functions, including killing the host cell, mediating bacterial uptake into the host cell (invasion), reprogramming actin to form a lesion in host cells underlying adherent bacteria, to mediate intracellular survival by affecting intracellular trafficking, or to provide polymerized actin mechanisms to propel microbes around inside host cells and into adjacent cells. Collectively, these examples represent many key microbial virulence mechanisms that have led to a much deeper understanding of both microbial pathogens and GTPase functions.

1 Introduction

There are approximately 100 pathogenic microbes that cause significant disease in humans, accounting for one-third of all deaths on the planet, in addition to many other pathogens that infect other mammals, animals, and plants. These pathogenic microbes possess many sophisticated virulence strategies that are designed to overcome generally effective host defense mechanisms that defend against the continual exposure to microbes [7]. Generally, these virulence mechanisms target one or more normal host cellular processes, and it is the collective action of these mechanisms that ultimately ends in disease.

The choice of host processes to target are numerous: signaling mechanisms, cell division, host immune response, phagocytosis, epithelial barrier integrity, chemotaxis, phagosome-lysosome fusion, etc.. Most virulence factors target a specific host molecule to mediate these effects. This entails direct contact of the bacterial virulence factor with the appropriate host molecule, which may be on the host cell surface or, in many cases, inside the host cell. Thus bacterial pathogens have developed various strategies to deliver their virulence factors, from binding to the cellular surface, being taken up by normal endocytic routes, and then escaping the membrane bound inclusion (many toxins use this route) to being injected directly into the host cell with specialized type III and type IV secretion systems [11, 14] and then targeting to the appropriate intracellular location. Recent knowledge in cell biology has advanced very rapidly, and much of this progress is due to the use of virulence factors as tools to study normal cellular processes. Indeed, the function of such key cytoskeletal regulators such as Rho, N-WASP, and Arp2/3 were discovered by using bacterial factors that modulate them.

An ideal virulence factor target should be one that controls one or more important cellular processes, and whose manipulation will provide the invading pathogen with a subsequent advantage for surviving and multiplying within the infected host. Thus the more “ideal” the host target, the more examples there are of virulence factors that aim to alter and/or disrupt such a target. One of the most extensively targeted cellular processes is the ability to alter cytoskeletal rearrangements, especially the subset of actin-based processes (as opposed to microtubules or intermediate filaments). The actin-based cytoskeleton has many important roles in eukaryotic cells, including cell motility, phagocytosis, and cell division, and is an essential process to eukaryotic cells. Not surprisingly, many bacterial pathogens go after the “master controls” of the cytoskeleton, the small GTP-binding proteins belonging to the Rho family of GTPases, and have devised many clever ways to activate, inactivate, modulate, and generally manipulate these important cellular regulators, ultimately using these mechanisms as part of their overall virulence strategy.

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Small GTPases

Although ATP serves as the main energy source within cells, many proteins (called G proteins) can bind and cleave GTP to regulate cellular processes and mechanisms. Generally, G proteins are divided into two groups, with a major family being the small G proteins or small GTPases (20–40 kDa).

Table 1 Small GTPase families and functions

Small GTPase family	General functions
Ras	Regulator of gene expression, cell division and transformation, MAP kinase cascade, cell proliferation and differentiation, apoptosis
Rho (includes Rac and Cdc42)	Modulators of actin cytoskeleton, activation of NADPH oxidase, stress fibers (Rho), lamellipodia and membrane ruffles (Rac), filopodia (Cdc42)
Rab	Intracellular vesicle targeting, docking, and fusion
Sar1/Arf	Vesicle membrane recruitment, including COPII (Sar1) and COPI, AP-1, and AP-3 (Arf)
Ran	Nucleocytoplasmic transport and microtubule organization

This family consists of over 100 members, being found in eukaryotes ranging from yeast to human. These break down into a further five subgroups or families: Ras, Rho, Rab, Sar1/Arf, and Ran [16]. Each family has generalized functions that are essential for normal cellular functions, including signaling, cytoskeletal rearrangements, vesicle targeting, nucleocytoplasmic transport, and microtubule organization (Table 1). (For a very comprehensive review on small GTPases and their functions, see [16].) Currently, four of these families (Ras, Rho, Rab, and Sar1/Arf) are targeted by microbial virulence factors, and, although not documented yet, the Ran family makes an attractive target to disrupt either nuclear transport or microtubule function by microbial pathogens.

All the small GTPases share a common mechanism by which they function, based on the ability to bind and cleave GTP (Fig. 1) [16]. In addition, this mechanism uses several other regulatory proteins, which results in a very finely controlled molecular switch that is used to modulate cell functions. A small GTPase is in the inactive form when it binds GDP. An upstream activation signal stimulates the dissociation of GDP followed by the binding of GTP, which leads to a conformational change enabling the activated GTPase to then bind to and activate downstream effectors (Fig. 1). The intrinsic GTPase activity of the small GTPase then cleaves GTP into GDP, which releases the bound effector, returning the GTPase back to its inactive form. Regulator proteins called GEFs (the guanine nucleotide exchange factors) assist the rate-limiting step of the GDP/GTP exchange by facilitating release of bound GDP followed by subsequent GTP binding, which displaces GEF. Thus GEFs

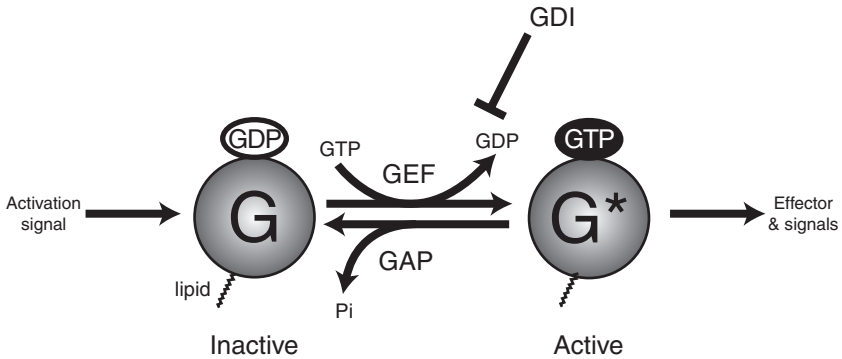


Fig. 1 Generalized mechanism of small GTPase regulation and activity. See text for details

“activate” small GTPases. Another regulator, called GAP (GTPase activating protein) stimulates the GTPase activity further (Fig. 1). Members of the Rab and Rac/Rho/Cdc42 family use an additional regulator called GDI (GDP dissociation inhibitor), which inhibits the dissociation of GDP from the small G protein, keeping the G protein in its (inactive) GDP-bound state. In addition to regulatory molecules that modulate activity, small GTPases are also covalently modified by lipids at their COOH termini, including farnesylation, geranylgeranylation, and prenylation (see [16] for details). Inhibitors that block these lipid modifications block G protein migration to the membrane, which inhibits activity. Collectively, all these regulators and modifications tightly control the activity of small G proteins, allowing the cell to rapidly turn on and off these key elements of cellular function. Not surprisingly, many microbial pathogens have also realized the power of manipulating small GTPases and their regulatory elements and target many G proteins, GAPs, GEFs, and GDIs.

The Rho family of small G proteins contains several family members, including several Rac and Rho members, plus Cdc42. These GTPases are generally thought to control actin cytoskeleton-based functions [10]. There are also numerous effectors upstream that trigger their activity (see [16] for details). In general, Rho family members control stress fibers in cells, which are long, extended bundles of actin that are easily visible along the basolateral plane of cultured mammalian cells. Rac family members regulate membrane ruffles and lamellipodia that occur at active areas of mammalian cell surfaces. Cdc42 binds N-WASP, which then activates the actin polymerization machinery Arp2/3, which mediates filopodia (fingers at the cell surface) formation, as well as other actin-based functions.

3 Small GTPases and Bacterial Virulence Strategies

As discussed above and seen throughout this volume, small Rho GTPases are utilized extensively by bacterial pathogens to usurp normal cellular processes as part of virulence. This is presumably because of their central role in cellular functions and their many diverse downstream effects. These virulence factors generally fall into two categories: toxins that bind to cellular surfaces and can be internalized into host cells and effector proteins that are injected via type III and type IV secretion systems directly into host cells, where they can then target appropriate G proteins. In general, the toxins kill the host cells (thus their name), whereas effectors modulate cellular effects that benefit the pathogen, such as mediating bacterial uptake into host cells (invasion). Each of these processes is discussed in extensive detail throughout the remainder of this volume, and thus the details of each of the processes will not be reiterated here. Instead, a more general overview of the processes as they relate to bacterial virulence is presented in the context of bacterial virulence strategies.

Perhaps the most daunting obstacle a pathogen faces when encountering a potential host is the epithelial barrier. At some points on the body such as skin, this barrier is so impermeable the only way through it is via a break (cut) or at the base of hair follicle sites. However, at other sites in the body such as most mucosal surfaces, the barrier must be more permeable, as the body needs to shunt nutrients and cells such as phagocytes back and forth. The tight seal between epithelial cells (tight junctions or zona occludens) is supported by a band of actin running around the peripheral apical surface of each cell. Because it is made of actin, small Rho GTPases play a key role in maintaining its integrity (and thus cell polarity). Not surprisingly, bacterial pathogens have developed various ways to disrupt this barrier to gain access to deeper tissue or cause diarrhea. Thus Rho GTPases make attractive targets for pathogens that require the breakdown of epithelial barriers. Such breakdown may inhibit the normal immune sampling mechanisms by disrupting the normal cytology, disrupting chemotactic recruitment of macrophages and neutrophils, disrupting normal nutrient uptake, and altering luminal fluid production. It is thought that triggering diarrhea enhances the removal of normal flora that normally compete with an incoming pathogen for limited nutrient supplies. Additionally, enhanced fluid secretion increases the spread of a diarrheagenic pathogen in the environment, allowing it to colonize many new individuals.

Mucosal surfaces are usually the first host surface pathogens contact. The ability of pathogens to successfully adhere to cellular surfaces is a key virulence

attribute [12]. Adherence is usually mediated by various bacterial adhesins such as fimbriae, pili, and afimbrial adhesins. Epithelial cells of the intestinal surface (and most other epithelial surfaces) form extensive networks of actin-based microvilli. Although this increases the cellular surface area, which enhances nutrient uptake, it is unclear whether this is a benefit or detriment to bacterial adherence. Nonetheless, some bacterial pathogens trigger microvillus disruption and surface rearrangements, including *Salmonella* species and the attaching and effacing pathogens such as pathogenic *E. coli* [6]. Because actin is the key regulator of microvilli, it is not surprising that these pathogens target small Rho GTPases [8] to disrupt microvilli. In addition, *Salmonella* is able to insert a phosphatidylinositol phosphate phosphatase (SigD) into the host cell, which “loosens” the cellular surface (presumably by altering the underlying cytoskeletal architecture), enhancing bacterial invasion [17]. Given their key role in cellular signaling, the ability to modulate a host cellular surface could be of significant benefit to a pathogen intent on adhering to an epithelial surface. This could include up- or downregulating one or more host cell proteins with which the pathogen might interact (see chapter by Duménil and Nassif, this volume). It could also include altering cellular surface function that may impact on the host’s response to a pathogen (such as affecting Toll-like receptor signaling normally used to detect pathogens). Finally, it could also include altering the underlying cytoskeletal architecture. For example, Rho GTPases modulate the ezrin/radixin/moesin proteins in leukocytes, which affects integrin, ICAM, L-selectin, and other surface markers, which ultimately affects leukocyte migration, T-cell interaction with antigen-presenting cells, apoptosis, and phagocytosis [13].

Perhaps one of the most remarkable attributes of pathogens that activate small G proteins is that they often also encode the machinery to turn the G proteins off once the process is complete. This has been worked out very well for *Salmonella* species (see chapter by Schlumberger and Hardt, this volume, and [8]). This pathogen uses a type III secretion system to inject at least three bacterial effectors [SopE (a GEF), SopE2 (also a GEF), and SigD], which activate Cdc42 and Rac to facilitate actin-based bacterial uptake and invasion. However, it uses the same secretion system to also insert SptP, a GAP that inactivates Cdc42 and Rac activity and returns the actin cytoskeleton to a near-normal state after bacterial invasion, while the bacteria remain inside a vacuole in the host cell.

The ability to enter into a host cell provides a unique opportunity for a pathogen. It allows it to enter a noncompetitive environment free from normal flora. (In the large bowel, there is such competition for nutrients that *E. coli*’s division time is about 24 h, compared to 20 min in rich broth, and it is in stationary phase in the gut). In addition, once inside a host cell, a pathogen

is privy to a rich nutrient and moisture source, free from circulating antibodies and patrolling macrophages and neutrophils. Many bacterial pathogens invade host cells, and nearly all do this by exploiting members of the Rho GTPase family [5]. Most, such as *Shigella* and *Salmonella*, do this by injecting effectors that modulate Rac and Cdc42 activity. Examples of pathogens that use Rho family-mediated invasion include *Bartonella*, *Brucella*, *Chlamydia*, *Listeria*, and *Pseudomonas*. Indeed, it is felt that nearly all invasive pathogens use such a strategy for invasion, except perhaps for *Rickettsia*, which seems to use a phospholipase activity to break into a cell.

Once inside a cell, some pathogens digest the vacuole surrounding them and escape into the cytoplasm, where they then polymerize cytosolic actin [4]. This polymerization event propels them around inside the host cell and enables them to spread into adjacent host cells without becoming extracellular. *Shigella*, *Listeria*, and some *Rickettsia* use such a mechanism as part of their virulence [4]. However, instead of activating small Rho GTPases, these pathogens actually mimic downstream components to achieve this actin rearrangement. The *Shigella* protein IcsA recruits and activates N-WASP, leading to Arp2/3 complex recruitment and actin polymerization. This thus replaces the function of Cdc42. The *Listeria* protein ActA activates Arp2/3 directly, thereby directly mimicking N-WASP's activity.

Another family of pathogens, including enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC), uses a type III system to insert effectors and their own receptors into epithelial surfaces. This results in a spectacular rearrangement of cellular actin and pedestal formation beneath the pathogen. Although Rho G proteins do not seem to be involved in this process [2], EPEC inserts its own protein (Tir) into host membranes, where it is tyrosine phosphorylated and binds the adaptor protein Nck, which then activates N-WASP and Arp2/3 complex recruitment [9]. The above examples indicate that modification or activation of Rho G proteins is not necessary, but instead mimics can be deployed to modulate actin dynamics.

For pathogens that invade and remain within a vacuole, it is critical to avoid lysosomal fusion (and subsequent bacterial death). Again, Rho GTPases (as well as Rabs) are involved in this process and other intracellular killing mechanisms, and inactivation of these may enhance intracellular survival (see chapter by Diebold and Bokoch, this volume). For example, *Legionella pneumophila* uses a type IV secretion system to insert an effector (RalF) into host cells that functions as a GEF to activate the small GTPase ARF, which modulates lysosomal fusion of the vacuoles containing bacteria [15].

Professional phagocytic cells are designed to use actin-mediated events to internalize pathogens and then destroy them in phagolysosomes. However, several pathogens including *Yersinia*, *Pseudomonas*, and EPEC have developed

strategies to avoid phagocytosis by blocking this process [3]. Given the central role of actin in phagocytosis, it is no surprise that these mechanisms involve pathways to disrupt normal actin-mediated processes (see chapter by Deckert et al., this volume).

Many bacterial toxins and effectors either activate or deactivate Rho GTPases for a variety of functions [1]. Often this activity is mediated by enzymatic modification of the G protein. The variety of effects modulated by these bacterial toxins is impressive and serves as a major arsenal for pathogenic microbes. Often killing the host cell is one outcome, but many more subtle effects have been noted, which are a significant focus of this volume. In addition, these effectors and toxins have become valuable tools to dissect normal cellular functions and are used extensively by cell biologists without realizing their natural role in biology.

4 Looking Ahead

Although many examples of virulence strategies that involve Rho GTPases have been documented, there are many, many more undiscovered ones. In fact, it is probable that for every normal and important cellular process, there is probably a pathogen that has designed a mechanism to exploit it. There is currently an explosion in identifying type III and type IV effectors that are inserted into host cells yet currently lack an identified host target. It is probable that, in addition to the Rho family, there are many intracellular pathogens that alter members of the Rab and Arf family to survive inside host cells; this is a field in its infancy. The extensive signaling mediated by the Ras family makes these ideal targets for virulence factors to reprogram cells. It is also likely that factors that alter Ran function and reprogram the nucleus will be identified.

It is apparent that there are several examples of bacterial virulence factors that specifically target members of the Rho GTPase family and their regulators. What is remarkable is the diverse effects such mechanisms have on virulence strategies, ranging from killing the host cell to mediating invasion and intracellular invasion. Because of the genetic plasticity and promiscuity of bacterial genetics, once a pathogen has found a successful virulence mechanism, this is rapidly passed on by plasmids, phage, and conjugation to other pathogens. This results in new combinations of virulence factors, which subsequently translate into new pathogens and emerging infectious diseases. Given the extent of bacterial modulators of Rho GTPases, there is no doubt that many more such effectors will be discovered. Also, because these GTPases

all use similar mechanisms for activation and regulation (Fig. 1), it is not hard to imagine how a pathogen might alter a virulence factor to work in a similar way on a different GTPase.

The ability to cause disease is a compilation of mechanisms that collectively reprogram and override the host, resulting in pathogen proliferation and spread. Because Rho GTPases play such a central role in normal cellular function, they are used by many successful bacterial pathogens as targets of effectors. As our knowledge increases about these mechanisms, so does our understanding of bacterial virulence. An added benefit is that these toxins and effectors also serve as excellent tools to understand basic cellular processes. This volume is a testimony to this rapidly expanding and important knowledge.

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