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6.1. Introduction

Extensive studies carried on Rho proteins have unveiled several layers of regulations of these small GTPases including posttranslational modifications (PTMs) (Hodge and Ridley, 2016; Popoff, 2014; Aktories, 2011). Rho GTPases are molecular switches that undergo a GTP/GDP-based cycle that is described in other chapters and reviewed in (Hodge and Ridley, 2016). Briefly, they bind and hydrolyze the guanosine triphosphate (GTP) into guanosine diphosphate (GDP) thereby oscillating between a GTP-bound active form and a GDP-bound inactive form (Wittinghofer and Vetter, 2011). The switch imposes conformational changes into two flexible loops or switch regions that are involved in effector binding and GTP hydrolysis. Transitions between both forms are controlled by guanine

nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and guanosine nucleotide dissociation inhibitors (GDIs) protein families (see Chapter 3 by Amin and Ahmadian). The GTP-bound form can contact several effector proteins at the interface of membranes to coordinate the spatiotemporal organization of protein complexes that transduce signals. A series of PTMs of the CAXX-box terminal part are essential to the function of small GTPases (Olson, 2016). This stabilizes the anchorage of the GTP-bound active form in membranes. For Rho GTPases, this commonly involves a first step of geranylgeranylation of the cysteine residue of the CAXX motif, depicted in Fig. 1, that is followed by the endoproteolytic removal of the last three amino-acids and subsequent carboxymethylation of the cysteine; see Liu *et al*. (2012) for review. In addition, a reversible palmitoylation of the Cys-178, located up-stream of the CAXX-motif, in Rac1 targets this GTPase to cholesterol-rich liquid ordered membrane microdomains, where it is stabilized in its active form to control actin cytoskeleton dynamic and thereby membrane organization (Navarro-Lérida *et al*., 2012).

Rho proteins are essential signaling hubs that belong to the superfamily of p21 Ras-related small GTPases. They share a high degree of amino-acid sequence identity that witnesses a high selective pressure on critical amino acids directly involved in the binding and hydrolysis of the guanine nucleotides, as well as their control of essential cellular functions (Olson, 2016; Hodge and Ridley, 2016). Apart from the highly conserved GTPase scaffold, Rho GTPase members display specific amino-acid sequences in particular in the switch-I region and at their carboxy-terminal end. At the difference of other small GTPases, Rho proteins display an insert region (amino acids 124–136 in RhoA). These variations of primary amino-acid sequence confer to Rho proteins their identity of interaction with regulators and effectors, as well as their specificity of localization at the membrane interface (Olson, 2016; Hodge and Ridley, 2016). In humans, there are 20 Rho GTPase members that can be subdivided into four evolutionary conserved clusters, comprising Rac, Rho, RhoH, and RhoBTB (Olson, 2016; Boureux *et al*., 2007) (see Chapters 1 and 2 by Fort and Aspenström). The three most studied Rho proteins, RhoA, Rac1, and Cdc42, are critical regulators of the architecture and dynamics of the actin cytoskeleton that sculpts cell shape and provides contractile forces, thereby conferring to the cells their capacity to adhere to the matrix, form junctions in cell monolayers, as well as migrate and phagocyte microbes (Ridley and Hall, 1992; Ridley *et al*., 1992; Nobes and Hall, 1995; Caron and Hall, 1998). Remarkably, Rho members are the target of PTMs catalyzed by an impressive array of bacterial and cellular factors discussed in this chapter.

Deconstructing our understanding of the regulation and functions of small Rho GTPase by means of studying bacterial toxins is a strategy that provides an

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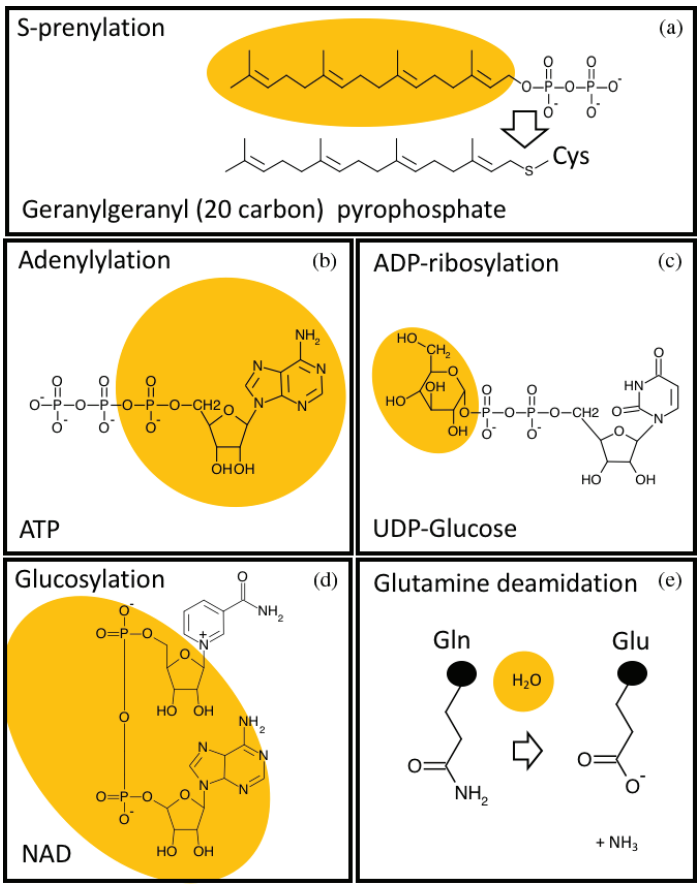

Figure 1. Examples of cosubtrates involved in PTMs. The figure depicts in orange the part of the molecule coupled to amino acid residues on the target: (a) This example of S-prenylation corresponds to the cross-linking of the 20-carbon backbone of geranylgeranyl to the cysteine residue of the CAXX-box motif. (b), (c), (d) ATP, NAD^+, and UDP-glucose cosubtrates involved in reactions of Adenylation (or AMPylation), mono-ADP-ribosylation, and glucosylation, respectively. (e) Reaction of glutamine deamidation.

invaluable source of information on these molecular switches (Visvikis *et al.*, 2010; Lemichez and Aktories, 2013). This prolific research in cellular microbiology has unveiled an unprecedented convergence of bacterial virulence toward host GTPases notably of the Rho family. The study of *C. botulinum* C3 exoenzyme was the pioneer investigation of Rho protein in the control of actin cytoskeleton (Chardin *et al.*, 1989; Paterson *et al.*, 1990). Here, we review this intimate cross talk

between bacteria and Rho proteins. This comprises newly described PTMs of small GTPases other than Rho proteins given that it broadens our knowledge of the repertoire of possibilities. In addition, we discuss examples of PTMs of Rho GTPases by the cellular machinery.

6.2. Post-translational modifications of small Rho GTPases

6.2.1. *Bacterial effector-mediated posttranslational modifications of Rho GTPases*

Modulation of Rho GTPase activity by PTMs is a strategy exploited by bacteria to breach host barriers and defenses (Lemichez and Aktories, 2013). They produce virulence factors that are either AB-type toxins, endowed with the capacity to enter cells via endocytosis and translocate their enzymatic part into the cytosol (Lemichez and Barbieri, 2013), or factors directly injected through syringe-like secretion systems into the cytosol by cell-bound bacteria, here referred to as injected effectors (Galan, 2009). PTMs of Rho proteins catalyzed by bacterial factors irreversibly activate or inactivate small GTPases and promote major pathophysiological outcomes (Lemichez and Aktories, 2013). The unregulated activation or inhibition of Rho proteins by bacterial factors can produce similar effects such as efficient disruption of epithelial and endothelial barriers (Lemichez and Aktories, 2013). Another example is provided by recent findings showing that bacteria-induced inhibition of RhoA or activation of Rac1 both trigger Caspase-1-mediated processing of pro-IL-1β (Xu *et al*., 2014; Aubert *et al*., 2016; Eitel *et al*., 2012; Diabate *et al*., 2015; Zhao and Shao, 2016). On the other hand, the bidirectional modulation of Rho GTPase activity by PTMs can produce antagonist effects. For example, although the activation of Rho proteins, notably Rac1, by Gram-negative enteric bacteria foster their entry into epithelial cells by conferring to epithelial cells phagocytic-like properties (Boquet and Lemichez, 2003), the inhibition of Rho proteins is a strategy used by *Yersinia* spp. or *Pseudomonas* to block phagocytosis (Popoff, 2014; Caron and Hall, 1998). Thus, the bidirectional modulation of Rho protein activities combined with a targeting of different combinations of small GTPases confer general and specific pathogenic features to bacteria.

A large repertoire of PTMs of Rho GTPases has been characterized over the past 20 years, clarifying the mode of action of essential virulence factors from several major human pathogens, such as *Clostridium* spp., *Yersinia* spp. and *Escherichia coli* (Popoff, 2014; Lemichez and Aktories, 2013; Aktories, 2011) (Table 1). As discussed below and reviewed in Aktories (2011), these PTMs

Table 1. Examples of PTMs of small GTPases induced by bacterial factors. Q: glutamine (Gln); T: threonine (Thr); S: serine (Ser); C: cysteine (Cys); N: asparagine (Asn).

PTMs	Modified amino-acids & targets	Examples of virulence factors	References
Deamidation	Q63 in RhoA (Q61 in Rac1/Cdc42)	CNF1 from *E. coli*	Flatau *et al.* (1997); Schmidt *et al.* (1997)
	N41 in Rh[illegible]	TecA from *B. cenocepacia*	Aubert *et al.* (2016)
Transglutamination	Q63 in RhoA (Q61 in Rac1/Cdc42)	DNT from *Bordetella* spp.	Masuda *et al.* (2000)
Glucosylation with UDP-glucose	T37 in RhoA (T35 in Rac1/Cdc42)	TcdA & TcdB from *C. difficile*	Just *et al.* (1995); Selzer *et al.* (1996)
Glycosylation with UDP-*N*-acetylglucosamine	T37 in RhoA (T35 in Rac1/Cdc42)	α-toxin from *C. novyi*	Selzer *et al.* (1996)
AMPylation	T37 in RhoA (T35 in Rac1/Cdc42)	VopS from *V. parahaemolyticus*	Yarbrough *et al.* (2009)
Endoproteolysis	C200 in RhoA	YopT from *Yersinia* spp.	Shao *et al.* (2003)
Phosphocholination	S79 in Rab1A (S77 Rab1B)	AnkX from *L. pneumophila*	Mukherjee *et al.* (2011)
Ubiquitin cross-linking	ADP-ribosylation of ubiquitin followed by phosphoribosylated-ubiquitin transfer to Rab33b	SdeA from *L. pneumophila*	Qiu *et al.* (2016); Bhogaraju *et al.* (2016)

irreversibly switch on or off Rho proteins. There is only one way to short-circuit the GTP/GDP cycle that consists in blocking the intrinsic GTPase activity. This can be achieved by PTMs of a critical glutamine (Q) residue that positions the water molecule for the nucleophilic attack of the γ-phosphate (Wittinghofer and Vetter, 2011; Aktories, 2011). PTMs go from a discrete deamidation of this glutamine residue into a glutamic acid (Flatau *et al*., 1997; Schmidt *et al*., 1997), up to the covalent attachment of bulky chemical groups, such as lysine and polyamines (Masuda *et al*., 2000) or the ADP-ribose moiety from NAD^+ (Lang *et al*., 2010) (Fig. 1). In contrast, PTMs of several critical amino-acid residues of the switch-I domain, or of the carboxy-terminal part of Rho protein, can block signal transduction mediated by Rho proteins. Modifications of the switch-I domain encompass by order of discovery mono-ADP-ribosylation (Chardin *et al*., 1989; Sekine *et al*., 1989), glucosylation (Just *et al*., 1995), endoproteolysis (Shao *et al*., 2003), and AMPylation earlier known as adenylylation (Yarbrough *et al*., 2009; Worby *et al*., 2009) (Fig. 1). Several amino-acid residues in the switch-I domain are the targets of PTMs catalyzed by bacterial effectors. This comprises Tyr-34, Thr-37, Thr-39, and Asn-41 of RhoA. For example, large glycosylating toxins from *Clostridia* target a threonine residue located at the middle of the switch-I domain of several Rho GTPases (Thr-35/37 for Rac1/RhoA) using either uridine diphosphate (UDP)-glucose or UDP-*N*-acetylglucosamine, as cosubtrates (Table 1). All known toxins modify only one specific amino acid of Rho but commonly several Rho members. Only, C3-like exoenzymes from several Gram-positive bacteria (Aktories, 2011) are relatively specific of a Rho GTPase, namely RhoA. Amino acids such as Asn-41 and Tyr-34 in RhoA undergo different types of PTMs catalyzed by unrelated bacterial factors. Indeed, Asn-41 can be either mono-ADP-ribosylated by C3-like exoenzymes or deaminated into an aspartic acid by TecA from *Burkholderia cenocepacia* (Aubert *et al*., 2016). Similarly, the Tyr-34 of RhoA can be either AMPylated by IbpA from *Histophilus somni* or glycosylated by PaTox from *Photorhabdus asymbiotica*, a toxin that uses UDP-*N*-acetylglucosamine as cosubstrate (Worby *et al*., 2009; Jank *et al*., 2013).

Taken collectively, this convergence of virulence toward a group of host proteins certainly highlights the critical function of Rho GTPases in host defenses for a broad array of host organisms and notably protozoan natural bacterial predators. Pathogens frequently combine the attack of Rho GTPases together with that of actin molecules. For example, virulent strains of *C. difficile* produce both a two-component toxin (CDT) that mono-ADP-ribosylates actin and large glucosylating toxins (TcdA and TcdB) that target RhoA, Rac1, and Cdc42 (Aktories *et al*., 2011). The Tc toxin from *Photorhabdus luminens* ADP-ribosylates Rho and actin thereby disrupting the architecture of the actin cytoskeleton into F-actin clusters (Lang *et al*., 2010). These

examples suggest that the targeting of both actin and Rho GTPases likely concurs to an efficient disruption the actin cytoskeleton. Nevertheless, a likely hypothesis is that such complementary virulence might also sign the importance of targeting several functions controlled by Rho GTPase apart from the control of actin cytoskeleton regulation.

Some pathogens inject into host cells an array of virulence effectors that display different biochemical activities toward various subsets of Rho proteins. This is probably best illustrated by *Yersinia pestis*, the agent of the bubonic plague, and other *Yersinia* species that trigger lymphadenopathy or acute enteritis. Here, virulence effectors targeting Rho proteins act in concert to freeze phagocytic function of macrophages. Indeed, *Yersinia* injects several Yop factors targeting Rho GTPases, comprising (i) the endoprotease YopT that cleaves the carboxy-terminal part of RhoA (Shao *et al*., 2003), (ii) YopE that displays a GAP-like activity on Rho proteins, comprising RhoA (Aili *et al*., 2006) and (iii) YpkA (YopO) that contains a Rho GDI-like domain and a kinase domain targeting heterotrimeric Gαq to produce an indirect inhibition of RhoA (Navarro *et al*., 2007; Prehna *et al*., 2006). Unexpectedly, inhibition of RhoA in macrophages leads in turn to an activation of pyrin inflammasome for IL-1β secretion and the induction of pyroptotic cell death (Xu *et al*., 2014). Remarkably, the bacterium injects another effector to tamper innate immune responses that would otherwise result from pyrin inflammasome activation (Trosky *et al*., 2008). Indeed, in nonintoxicated cells, RhoA activates the serine/threonine protein kinase C-related kinases (PRK or PKN isoforms 1 and 2) that bind to and phosphorylate pyrin to promote its trapping by 14-3-3. This sequestration keeps pyrin inflammasome silent (Park *et al*., 2016). Interestingly, *Yersinia* spp. opposes the induction of pyrin inflammasome by coinjecting YopM (Chung *et al*., 2014, 2016; Schoberle *et al*., 2016). Molecularly, YopM binds to PRK to promote a RhoA-independent phosphorylation of pyrin that restores its interaction with 14-3-3 for inactivation (Aubert *et al*., 2016; Chung *et al*., 2016). PTM-driven activation of Rac1 by the CNF1 toxin from *E. coli* also triggers caspase-1 mediated IL-1β secretion (Diabate *et al*., 2015). Collectively, these findings point for inflammasome complexes as sensors of RhoA and Rac1 activities.

6.2.2. *Extended diversity of small GTPase PTMs*

It is intriguing why GTPases (small, heterotrimeric, or large GTPases, such as elongation factor EF) are frequent substrates of various toxins and injected effectors (Lemichez and Barbieri, 2013). Bacterial effectors do not induce a unique type of modification but selectively modify GTPases through diverse enzymatic reactions. Novel toxin-dependent PTMs of small GTPases of the Rab subfamily

have been recently uncovered (Table 1). It is interesting to briefly discuss these PTMs, considering the diverted use of enzyme reactions found in nature. Indeed, the control of small GTPases by injected virulence effectors is not a peculiarity of Rho proteins. For example, the mono-ADP-ribosyl transferase activity of ExoS from *Pseudomonas aeruginosa* inactivates several Ras small GTPases; among them are Ras, RalA, as well as Rab5 and several other Rab members (Fraylick *et al*., 2002). The subfamily of Rab GTPases are critical regulators of membrane trafficking and thereby targets of choice for bacteria that thrive in intracellular compartments (Wandinger-Ness and Zerial, 2014; López de Armentia *et al*., 2016). The activity of ExoS from *Pseudomonas aeruginosa* is, for example, essential for the bacterium to avoid the reaching of lytic compartments once it gets internalized into epithelial cells (Angus *et al*., 2010). Exciting new studies unravel that bacterial effectors from *Legionella pneumophila* modify various cellular substrates including Rab GTPases, notably Rab1 family members. This intracellular pathogen injects into host cells more than 300 effectors that confer to bacteria versatile capacities to replicate in a broad number of host species, notably the fresh water amoebas, natural hosts, or humans leading to Legionnaire's disease. Bacteria replicate in a *Legionella* containing vacuole (LCV) at the membrane of which it recruits Rab1 family members in order to hijack vesicles derived from the Golgi and the endoplasmic reticulum. The injected virulence effector AnkX catalyzes a new type of PTM. This consists in the transfer of the phosphocholine moiety of cytidine diphosphorylcholine (CDP) on the Ser-79 of Rab1A (S76 in Rab1B) that is located in the switch-II domain (Mukherjee *et al*., 2011). In the host cell, AnkX produces an enlargement of early endosomes and impairs alkaline phosphatase secretion as does expression of Rab1 S79A. In addition, Rab1 is the target of the glutamine synthetase adenylyl transferase (GS-ATase)-like domain of DrrA (SidM) that AMPylates Tyr-77 of Rab1A (Y80 in Rab1B) to promote its activation (Müller *et al*., 2010). This indicates that several amino-acid residues in the switch-II can be modified to promote the activation of a small GTPase. Interestingly, the phosphocholination and AMPylation of Rab1 can be reverted by the action of Lem3 and SidD effectors, respectively (Tan and Luo, 2011; Tan *et al*., 2011). The PTMs of Rab GTPases catalyzed by injected effectors from *Legionella* contrast with known PTMs of Rho proteins that are irreversible. This highlights the importance of keeping on and off Rab1 cycling to successfully hijack its functions. This idea is reinforced by the finding that the GDI displacement and GEF domains of DrrA can work cooperatively with the GAP activity of LepB to foster the recruitment and activation of Rab1 family members to the LCV (Ingmundson *et al*., 2007). How precisely these factors cooperate to corrupt the spatiotemporal cycling of Rab GTPases is a challenging question to be addressed. One element, brought to our

comprehensive view is that the phosphocholination of Rab tamed the GTP/GDP cycle by blocking the GEF activity of the host onnecdenn (Dennd1A) protein, while allowing the binding of GEF-domain of DrrA to promote Rab1 cycling to the LCV (Mukherjee *et al*., 2011).

The repertoire of PTMs of small GTPases has been extended recently with the description of an atypical cross-linking of phosphoribosyl-ubiquitin on Rab33b. This PTM is catalyzed the SidE effector family from *Legionella pneumophila* (Qiu *et al*., 2016; Bhogaraju *et al*., 2016). SdeA contains several domains notably a phosphodiesterase (PPE) and a mono-ADP-ribosyltransferase (mART). SdeA catalyzes the ADP-ribosylation of the Arg-42 of ubiquitin using NAD^+ as cosubstrate (Qiu *et al*., 2016; Bhogaraju *et al*., 2016) (Fig. 1). This PTM of ubiquitin is followed by the cleavage of the phosphodiesterase bond of mono-ADP-ribosylated ubiquitin to produce phosphoribosyl-Ub (pUb) or the transfer of pUb to serine residues in Rab33b (Bhogaraju *et al*., 2016). Canonical reaction of ubiquitination involves a first step of ubiquitin activation by an E1 enzyme followed by the transfer of ubiquitin from E1 to an E2 enzyme, prior E3-mediated direct or indirect cross-linking of ubiquitin to target proteins (Swatek and Komander, 2016). The phosphoribosyl-Ub poisons all conventional transfer reactions of ubiquitin. It works in concert with the amino-terminal deubiquitinase domain DUB of SdeA to disengage host protein modulation by ubiquitination (Bhogaraju *et al*., 2016). The freezing of the host ubiquitination machinery likely plays a dominant role, over Rab modification, in term of virulence.

6.2.3. *Host reaction to bacteria-mediated PTM of Rho GTPases*

The study of Rho GTPase activation by the CNF1 toxin from pathogenic *Escherichia coli* has unveiled a new mode of regulation of Rho proteins by ubiquitin-mediated proteasomal degradation (Doye *et al*., 2002; Mettouchi and Lemichez, 2012). The CNF1 toxin is produced by pathogenic strains of *E. coli* of the phylogenic group B2. These bacteria reside in the normal gut flora of healthy human carriers while promoting extraintestinal infections such as urinary tract infection and bacteremia (Welch, 2016; Buc *et al*., 2013). The CNF1 toxin is a paradigm of deamidase toxin and virulence effector targeting Rho GTPases found in a broad array of bacterial pathogens. CNF1 catalyzes a point mutation of Rho GTPases. This consists in a deamidation of the Gln-63 into a glutamic acid (61 in Rac1 and Cdc42) (Flatau *et al*., 1997; Schmidt *et al*., 1997; Lerm *et al*., 1999). Mutation of Gln-63/61 of RhoA/Rac1 impairs the intrinsic GTPase activities thereby turning on permanently Rho proteins (Flatau *et al*., 1997; Schmidt *et al*., 1997; Lerm *et al*.,

1999). Cells can react to this unregulated activation of Rho GTPases by promoting their polyubiquitination for targeting to degradation by the 26S proteasome (Doye *et al*., 2002). The HECT-domain and ankyrin-repeats containing E3 ubiquitin protein ligase 1 (HACE1) catalyzes the polyubiquitination of activated Rac1 on Lys-147 (Castillo-Lluva *et al*., 2013; Torrino *et al*., 2011). The depletion of HACE1 leads to an increase of total and active Rac1 cellular levels in intoxicated but also nonintoxicated cells (Castillo-Lluva *et al*., 2013; Torrino *et al*., 2011). HACE1 is therefore a critical element in the control of (i) Rac1-dependent NADPH oxidase-mediated production of reactive oxygen species, (ii) cyclin-D1 expression and cell cycle progression and (iii) autophagic clearance of protein aggregates (Daugaard *et al*., 2013; Rotblat *et al*., 2014; Zhang *et al*., 2014).

These guard functions of HACE1 on cell homeostasis and the control of Rac1-mediated cell cycle progression likely account in part for its critical tumor suppressor function (Zhang *et al*., 2007). Moreover, HACE1 regulates the tumor necrosis factor (TNF) receptor-1 (TNFR-1) signaling by shifting the balance of TNF signaling toward apoptosis instead of necroptosis and the ensuing inflammation (Ellerbroek *et al*., 2003). Mice knocked out for HACE1 display colitis when the intestinal epithelium barrier is compromised by treatment with the chemical irritant (DSS) and show a significant lower resistance to infection by *Listeria monocytogenes* (Tortola *et al*., 2016). A growing number of studies have established the importance of ubiquitin and proteasome systems (UPSs) in the control of Rho protein function (Table 2). Specific degradation of Rho proteins secures the spatial control of Rho activities and adapts the cellular levels of Rho to directly impact the extent of Rho proteins entering the GEF/GAP cycle (Wang *et al*., 2003; Chen *et al*., 2009; Torrino *et al*., 2011).

6.3. Cellular PTMs of Rho GTPases

Rho proteins are highly conserved from protozoan to humans. Most likely, the cellular PTMs of small GTPases represent a necessary adaptation to increasing levels of complexity found in multicellular organisms (Hodge and Ridley, 2016). Initially, Rho GTPases have been identified as membrane proximal regulators of the actin cytoskeleton in response to engagement of growth factors and G protein-coupled proteins' receptors, as well as integrin matrix-adhesion receptors (Ridley and Hall, 1992; Ridley *et al*., 1992; Nobes and Hall, 1995). The articulation of signals between receptor-coupled kinases and small GTPases involves the phosphorylation of GEF factors and the interplay of pleckstrin homology domains with phosphoinositides (Hodge and Ridley, 2016). As exemplified in Table 2 and reviewed in Hodge and Ridley (2016), several kinases can directly phosphorylate

Table 2. Examples of PTMs of Rho GTPases catalyzed by cellular factors.

GTPases	PTMs & amino acid targets	PTM Effects	Enzymes	References
Rac1	Phosphorylation T108	Induces nuclear translocation	ERK	Tong *et al.* (2013)
	Phosphorylation Y64	Inhibition	Src and FAK	Chang *et al.* (2011)
	Phosphorylation S71	Inhibits GTP binding	AKT	Kwon *et al.* (2000)
	Ubiquitylation K147	Proteasomal degradation	HACE1	Torrino *et al.* (2011); Castillo-Lluva *et al.* (2013)
	Ubiquitylation K166	Proteasomal degradation of S71 phosphorylated Rac1 (also Rac3)	SCF-FBXL19	Zhao *et al.* (2013)
	Sumoylation (carboxy-terminal region)	Stabilizes the GTP-bound form	PIAS3	Castillo-Lluva *et al.* (2010)
RhoA	Phosphorylation S188	GDI interaction & impaired proteasomal degradation	PKA, PKG, PKC (also T127), SLK	Lang *et al.* (1996); Ellerbroek *et al.* (2003); Su *et al.* (2013); Guilluy *et al.* (2008)
	Ubiquitylation K6, K7 and K51	Proteasomal degradation	Smurf1 and Smurf2	Ozdamar *et al.* (2005); Lu *et al.* (2011); Bryan *et al.* (2005)

(*Continued*)

Table 2. (*Continued*)

GTPases	PTMs & amino acid targets	PTM Effects	Enzymes	References
	Ubiquitylation	Proteasomal degradation	Cul3-BACURD	Chen *et al.* (2009)
	Ubiquitylation K135	Degradation	SCF-FBXL19	Wei *et al.* (2013)
	Serotonylation Q63 for RhoA	Activation	Transglutaminase	Walther *et al.* (2003)
	Nitration Y34	Activation by decreasing GDP binding	Addition of a nitro group from peroxynitrite to tyrosine residue	Rafikov *et al.* (2014)
Cdc42	Phosphorylation Y64	Inhibition by promoting association with GDI	Src	Tu *et al.* (2003)
	Phosphorylation S185	Inhibition by promoting association with GDI	PKA	Forget *et al.* (2002)
RhoU/ WRCH1	Phosphorylation Y254	Inhibition by relocalization to endosomal membrane	Src	Alan *et al.* (2010)
	Ubiquitylation K177, K248	Proteasomal degradation	Rab40A-Cullin5	Dart *et al.* (2015)

Rho GTPases. Despite progress made, much remains to be uncovered on PTMs of Rho GTPases if one considers the catalogue of PTMs defined by proteomic that encompass phosphorylation, ubiquitination, methylation, acetylation, and succinylation (http://www.phosphosite.org).

6.3.1. *Cellular and bacterial effector shared PTMs of Rho GTPases*

Human Fic-domain containing huntingtin yeast-interacting protein E (HYPE) displays *in vitro* and *in vivo* adenylylation activity toward RhoA, Rac1, and Cdc42 (Worby *et al.*, 2009). The cellular activity of HYPE is probably highly regulated in cells considering that its ectopic expression does not produce a disruption of actin cytoskeleton and a rounding of cells as does the immunoglobulin-binding protein A (IbpA) from *Histophilus somni* (Worby *et al.*, 2009). Note that IbpA also AMPylates LyGDI and blocks its phosphorylation by Src tyrosine protein kinase (Yu *et al.*, 2014). Mammalian transglutaminases catalyze the serotonylation of the Gln-63 of RhoA leading to its permanent activation (Walther *et al.*, 2003). Serotonin is a neurotransmitter in the central and peripheral nervous system and a ubiquitous hormone synthetized by tryptophan hydroxylase isoenzymes to control vasoconstriction and platelet function. Mice deficient in peripheral serotonin synthesis exhibit impaired hemostasis with high risk of thrombosis and thromboembolism, while the structure of platelets is not affected. Serotonylation of RhoA and other small GTPases promotes the exocytosis of α-granules from platelets by a receptor-independent mechanism thereby favoring their adherence to tissue lesions.

6.3.2. *Unique cellular PTMs of Rho GTPases*

In addition to the control of GTP/GDP cycle and membrane/cytosol shuttling, cells can modulate Rho GTPases by phosphorylation (Table 2). As discussed below, phosphorylation occurs both in the carboxy-terminal part and in the switch-II domain of small GTPases thereby controlling their cellular localization and guanine nucleotide loading, respectively. Although phosphorylation can be rapidly reverted, little is known on phosphatases that contribute to this balance. In contrast, progress has been made in identifying kinases targeting Rho proteins.

Two amino-acid residues in the switch-II domain of Rac1 can be phosphorylated thereby stabilizing the GDP-bound form. These PTMs comprise the phosphorylation of Ser-71 of Rac1 mediated by AKT; and Tyr-64, by Src or FAK (Chang *et al.*, 2011; Tu *et al.*, 2003; Kwon *et al.*, 2000). The phosphorylation of

Rac1 by Src modulates the spreading of cells on fibronectin (Chang *et al*., 2011). Expression of the phosphonull mutant Rac1-Y64F increases the pool of GTP-bound Rac1 and the spreading area of cells. Phosphorylation of amino-acid residues in the carboxy-terminal region of Rho proteins can modulate their localization. For example, the ERK-mediated phosphorylation of Rac1 on Thr-108 promotes its translocation into the nucleus, thereby compartmentalizing Rac1 away from components of its signaling cascade to down-modulate its activity (Tong *et al*., 2013). PTMs of Rho GTPases also contribute to modify their surface of interaction with regulatory proteins and effectors. Phosphorylation of Ser-188 of RhoA, among Rho phosphorylations, provides us with such an example (Lang *et al*., 1996) (Table 2). In several cell types, the broad signaling cyclic nucleotides cAMP or cGMP promote a PKA or PKG-dependent phosphorylation of RhoA on Ser-188 (Lang *et al*., 1996; Ellerbroek *et al*., 2003; Rolli-Derkinderen *et al*., 2005). PKA also phosphorylates the equivalent Ser-185 in Cdc42 that is not present in Rac1 (Forget *et al*., 2002). Phospho-Ser-188 can promote variations in the repertoire of activated effectors by excluding ROCK as compared to mDIA-1 and PKN (Nusser *et al*., 2006). In addition, phosphorylation of RhoA increases its capacity to interact with GDI, thereby altering its accessibility for activation and protects RhoA from proteasomal degradation mediated by Smad ubiquitylation regulatory factor 1 (Smurf1) E3 ubiquitin ligase (Forget *et al*., 2002; Rolli-Derkinderen *et al*., 2005). In contrast, phosphorylation of Rac1 on Ser-71 promotes its degradation by SCF-FBXL19 (Zhao *et al*., 2013). RhoA and Rac1 provide examples of cross talk between phosphorylation and ubiquitin-mediated proteasomal degradation.

While ubiquitination of Rho GTPases and notably Rac1 promotes their degradation, the SUMOylation of Rac1 carboxy-terminal-located lysines provides us with an example of cellular PTM that reinforces the stability of the GTP-bound form (Castillo-Lluva *et al*., 2010).

It is of great importance to further define how cellular PTMs of Rho GTPases contribute to their essential function in physiology. For example, the knockdown of HACE1 predisposes mice to colonic inflammation and cancer onset during aging (Tortola *et al*., 2016; Zhang *et al*., 2007). Yet to be demonstrated, growing evidence support a critical role of this regulation to tone down the oncogenic and inflammatory potential of Rac1 (Daugaard *et al*., 2013; Mettouchi and Lemichez, 2012; Goka and Lippman, 2015; Andrio *et al*., 2017).

6.3.3. *Regulation of atypical Rho GTPases by PTMs*

Some members of the Rho protein family, referred to as atypical small GTPases, display high intrinsic GDP exchange activities or substitutions in the GTPase

domain, which alter their capacity to hydrolyze the GTP. For these GTPases that escape to the canonical switch regulation by GEF/GAP factors, PTMs are a prominent way to regulate their signaling via compartmentalization and stability. RhoU (also known as WRCH1) provides us with an example of atypical Rho GTPase regulated both by phosphorylation and ubiquitylation. Although RhoU belongs to the Cdc42 branch of small Rho GTPases, it is thought to be constitutively active (Saras *et al*., 2004). This critical regulator of focal-adhesion dynamics is controlled by the kinase activity of Src, which catalyzes the phosphorylation Tyr-254 (Alan *et al*., 2010). Such modification of the carboxy-terminal part of RhoU promotes a shift in its subcellular localization from the plasma membrane to endosomal membranes, that is concomitant with a reduction of active protein levels likely through the segregation of RhoU from its GEF factors. In addition, RhoU is polyubiquitylated on Lys-177 and Lys-248 (Dart *et al*., 2015). This involves a recognition of Rab40A by RhoU. Over-expression of Rab40A promotes RhoU ubiquitylation and reduces its cellular level. By analogy with known homologues in *Xenopus*, control of RhoU stability by Rab40A likely involves an ElonginB/C and Cullin5 E3 ubiquitin ligase complex.

6.4. Conclusions

Despite their flexible regulation by GTP/GDP-based switches, the small Rho GTPases are controlled by PTMs that engage or disengage GTPase signaling in a dominant manner. A large spectrum of biochemical modifications of these GTPases has been unveiled going from deamidation of glutamine and arginine residues up to the addition of bulky chemical groups. These posttranslational modifications affect critical amino-acid residues of both switch-I and II domains and the carboxy-terminal region. The posttranslational modifications of Rho GTPases by virulence factors is a strategy widely exploited by bacterial pathogens to disrupt or usurp cell signaling, thereby conferring pathogens a better fitness. Cells can also react to PTMs of Rho GTPases by inducing inflammasome signaling or limit activation of these GTPases via their degradation. PTMs catalyzed by virulence effectors from *L. pneumophila* let us glimpse other types of modifications of Rho GTPases to be uncovered. This rich cross talk between bacterial virulence effectors and small GTPases continues to unveil critical elements of cell signaling especially those implicated in human diseases, such as infection, inflammation, and cancer.

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