Cell Death and Differentiation (1998) 5, 720–728

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Review

Bacterial toxins and the Rho GTP-binding protein: what microbes teach us about cell regulation

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Received 22.12.97; revised 31.3.98; accepted 13.5.98
Edited by R.A. Knight

Abstract

In the present review activities of two bacterial toxins, *Clostridium botulinum* exoenzyme C3 and *Escherichia coli* CNF1, both acting on the GTP-binding protein Rho are analyzed. Proteins belonging to the Rho family regulate the actin cytoskeleton and act as molecular switches in a number of signal transduction pathways. C3 and CNF1 have opposite effects on Rho thus representing useful tools for studies on cell division, cell differentiation and apoptosis.

Keywords: Rho; C3; CNF1; cell regulation

Abbreviations: CNF, cytotoxic necrotizing factor; GEF, guanine exchange factor; GAP, GTPase activating protein; LPA, lysophosphatidic acid; ROK, Rho kinase; PIP2, phosphatidyl-inositol-4,5-phosphate; PI4-P, phosphatidyl inositol 4 phosphate; ECM, extracellular matrix; SRE, serum responsive element; SRF, serum response factor

Introduction

Almost 10 years separate the discovery of a toxin which exerts an inhibitory activity on the p21 Rho small GTPase: the *Clostridium botulinum* exoenzyme C3 (Aktories *et al*, 1987; Rubin *et al*, 1988; Chardin *et al*, 1989) from that of a toxin which activates the same GTP-binding protein: the cytotoxic necrotizing factor 1 (CNF1) from *Escherichia coli* (Flatau *et al*, 1997; Schmidt *et al*, 1997). Exoenzyme C3 and CNF1 are now major tools for laboratories working on the actin cytoskeleton and signal transduction.

Aims of the present review are (i) to analyze the activities of these two bacterial toxins having opposite effects on the GTP-binding protein Rho; and (ii) to examine how these toxins can provide clues for explaining roles played by Rho in cell division, cell differentiation or apoptosis.

The GTP-binding protein Rho: a target for bacterial toxins

Rho protein (Madaule and Axel, 1985), discovered shortly after Ras (Chang *et al*, 1982), and YPT1 (a Rab-like GTPase from *Saccharomyces cerevisiae*) (Gallwitz *et al*, 1983), belong to a family of regulatory molecules now grouped under the name of 'Ras superfamily'. This superfamily encompasses three main groups of proteins: Ras, Rho and Rab which differ according to their sequence homology and their function. A certain number of other GTP-binding molecules (Arf and Ran) which harbour similarities with Ras are now linked to this superfamily of proteins (Zerial and Huber, 1995). The Ras group (Ras, Rap and Ral) is implicated in signal transduction of mitogenic signals, the Rho, Rac and Cdc42 subgroup regulates the F-actin cytoskeleton and the Rab subfamily (Rab 1 to Rab 30) controls intracellular traffic (Downward, 1990). Small GTP-binding proteins are under an active form, and are thus able to trigger a cascade of signalling events when they are associated with GTP. Linked to GDP, they are in the resting state. An activated small GTP-binding protein becomes inactive by hydrolyzing GTP into GDP. GTP hydrolysis by small GTP-binding proteins alone is normally exceedingly slow. In association with a protein named GTPase activating protein (GAP), GTP hydrolysis is very rapid. Activation of small GTP-binding proteins is due to the removal of their bound GDP. The protein which performs this task is the guanine exchange factor (GEF). The simple removal of GDP from the small GTP-binding protein allows binding of GTP since there is a large excess in cells of GTP over GDP. Two polypeptide domains change their conformation in small GTP-binding proteins when the molecule is associated either with GTP or GDP. These polypeptides are called switches (Milburn *et al*, 1990). Switch 1 (residues 30 to 39 in Ras, 32 to 41 in Rho) corresponds to the Ras polypeptide contacting its downstream effector (in the case of Ras it is Raf) whereas switch 2 (residues 60 to 76 in Ras, 62 to 78 in Rho) is implicated in the GTP to GDP hydrolysis.

Rho is mostly localized in the cytosol, associated with a molecule (guanine dissociation inhibitor GDI) which maintains its conformation in the inactive form (linked to GDP) (Fukumoto *et al*, 1990). When a growth factor (derived from a lipid, lysophosphatidic acid (LPA)) binds to its receptor (belonging to a family of receptors acting through heterotrimeric G proteins), it activates Rho via the Rho exchange factor at the level of the membrane. This mechanism and its precise localization are still poorly elucidated. Activated-Rho has two main targets for the regulation of the actin cytoskeleton: a serine-threonine kinase named Rho kinase (ROK) (Matsui *et al*, 1996; Ishizaki *et al*, 1996) and a kinase inducing, by phosphorylation (on position 5 of the inositol ring), the formation of

phosphatidyl-inositol-4,5-phosphate (PIP2) from phosphatidyl inositol 4 phosphate (PI4-P) (Chong *et al*, 1994; Ren *et al*, 1996). By regulating these two kinases, Rho might control the actin cytoskeleton by three mechanisms: (i) by acting on Rho kinase, it will provoke the bundling of actin filaments by directly (Amano *et al*, 1997) or indirectly (via phosphorylation of the myosin light chain phosphatase resulting in the inhibition of this enzyme) (Kimura *et al*, 1996) phosphorylating the myosin type 2 light chain allowing these molecules to associate with actin filaments and thereby provoking contractility (Fujihara *et al*, 1997); (ii) by locally raising the PIP2 concentration, Rho activates molecules bridging actin filaments and cell membrane-associated proteins such as vinculin (Gilmore and Burridge, 1996) ezrin, moesin or radixin (ERM group) (Hirao *et al*, 1996) and (iii) probably by provoking a PIP2 dependent actin polymerization (as described for Rac; Hartwig *et al*, 1995) by uncapping actin filament barbed ends (where addition of new actin subunits occurs). By these three mechanisms, Rho will allow extension of the cell surface (also called cell spreading). According to Cramer and Mitchison (1995), cell spreading results from actin polymerization at the cell periphery but also from the association of actin and myosin which induces cell contractility.

Rho also induces, by a mechanism implicating ezrin (a protein belonging to the ERM group), the formation of focal adhesion contacts (Mackay *et al*, 1997). Focal contacts are structures by which cells are anchored to the extracellular matrix (ECM) via integrins. As we will see below, anchoring to ECMs through integrins is an indispensable step for the generation of intracellular signals leading to multiplication, differentiation or apoptosis.

In addition to activities on cell spreading and formation of focal contacts, Rho exhibits other functions. One is relative to the regulation of endocytosis. Rho-GTP has an inhibitory effect on the formation of clathrin-coated vesicles, implicated in receptor-mediated endocytosis (Lamaze *et al*, 1996). Conversely, Rho-GTP seems to favour pinocytosis (Schmalzing *et al*, 1996), another system of endocytosis, but requiring no clathrin coat. Another additional function of Rho is its activity in the progression of cell cycle (Olson *et al*, 1995).

How can toxins acting on Rho give us clues concerning the role of these GTP-binding proteins in cell regulation? We will describe first the structure and function of two toxins acting on Rho, exoenzyme C3 from *C. botulinum* and CNF1 from *E. coli*. Then we will examine the cell activities of these toxins.

Exoenzyme C3 and CNF1: two bacterial toxins with opposite effects on the GTP-binding protein

C3 is produced by some strains of *C. botulinum* (serotypes C and D) in addition to C2 toxin and neurotoxins (Rubin *et al*, 1988). C3 is not a true toxin but the 25 kDa enzymatic moiety of a toxin. C3 activity on whole cells is difficult to assay since it cannot enter directly into the cytosol. To obviate this problem several toxins, called chimeric, have been prepared by genetic fusion associating C3 with the cell binding and membrane translocating polypeptides of diphtheria or *Pseudomonas aeruginosa* exotoxin A (Aullo *et al*, 1993; Boquet *et al*, 1995). Exoenzyme C3 is an ADP-ribosyltransferase which hydrolyzes NAD into ADP-ribose and nicotinamide and covalently links ADP-ribose to Rho Asparagine 41. Although asparagine 41 of Rho is in the switch 1 region (effector domain of Rho) (Sekine *et al*, 1989), it does not modify the architecture of the Rho effector domain sufficiently to block the interaction of Rho with the downstream effector ROK. However, ADP ribosylation of Rho blocks the translocation of Rho to the membrane resulting in the inhibition of Rho activity (Fujihara *et al*, 1997) (Figure 1).

CNF1 is a 110 kDa toxin produced by certain pathogenic strains of *E. coli* (Donelli and Fiorentini, 1997). This toxin causes necrosis when injected in rabbit skin and multinucleation in cultured cells (Caprioli *et al*, 1983). In cultured cells, the toxin mainly induces the formation of actin stress fibers and promotes cell spreading (Fiorentini *et al*, 1988, 1995) mimicking effects obtained by microinjection of the dominant active mutant of Rho (Paterson *et al*, 1990). CNF1 acts on Rho by a new mechanism for a bacterial toxin. Indeed, CNF1 will make Rho permanently active without the necessity for its interaction with the Rho exchange factor. After CNF1 modification, Rho loses its ability, both intrinsic or mediated by GAP, to hydrolyse GTP into GDP.

The loss of ability of Rho and RhoGAP to hydrolyse GTP into GDP is the result of an enzymatically mediated Rho protein modification, induced directly on the GTP-binding protein by CNF1. Indeed, CNF1 specifically acts on an important residue of the Rho switch 2 domain: glutamine 63 (Q63). Rho Q63 corresponds to Q61 of Ras. Glutamine 63 of Rho is implicated in the binding of a water molecule required for hydrolysis of the GTP gamma phosphate (Rittinger *et al*, 1997). Rho Q63 alone cannot stably maintain the water molecule during the transition state explaining the poor intrinsic GTP hydrolyzing activity of Rho (Rittinger *et al*, 1997). Associated to RhoGAP, which introduces an arginine residue (GAP arginine 85) close to Rho Q63, there is stabilization of Rho Q63 for the binding of the water molecule during the transition state resulting in a considerable acceleration of GTP hydrolysis (Rittinger *et al*, 1997). Q63 is thus a pivotal residue for Rho deactivation. Mutation of Q63 into another amino-acid decreases or totally blocks the GTPase activity of Rho and Rho associated to RhoGAP. We and others have shown that CNF1 exhibits a catalytic deamidase activity specific for Rho glutamine 63 (Flatau *et al*, 1997; Schmidt *et al*, 1997). Modification of Q63 to E63 in Rho blocks the intrinsic and GAP-stimulated hydrolysis of GTP resulting in the permanent activation of the GTP-binding protein (Flatau *et al*, 1997; Schmidt *et al*, 1997) (Figure 2).

C3 and CNF1 are invaluable tools for studying how Rho is involved in cell regulation

C3 effects on cultured cells brought the first indication that Rho could be implicated in the regulation of the actin

cytoskeleton. Treatment of cells with C3 (at high concentrations to force the penetration of the enzyme into cells) induces a selective disorganization of actin stress fibers (Chardin *et al*, 1989) (Figure 3). The role of Rho in the control of cytoskeleton organization was then demonstrated by microinjection into cells of Rho either mutagenized (to make it permanently active) or modified *in vitro* by C3. C3 has been used to demonstrate that Rho controls smooth muscle contractility (Fujihara *et al*, 1997) and very recently it has been shown that blood pressure could be controlled mostly

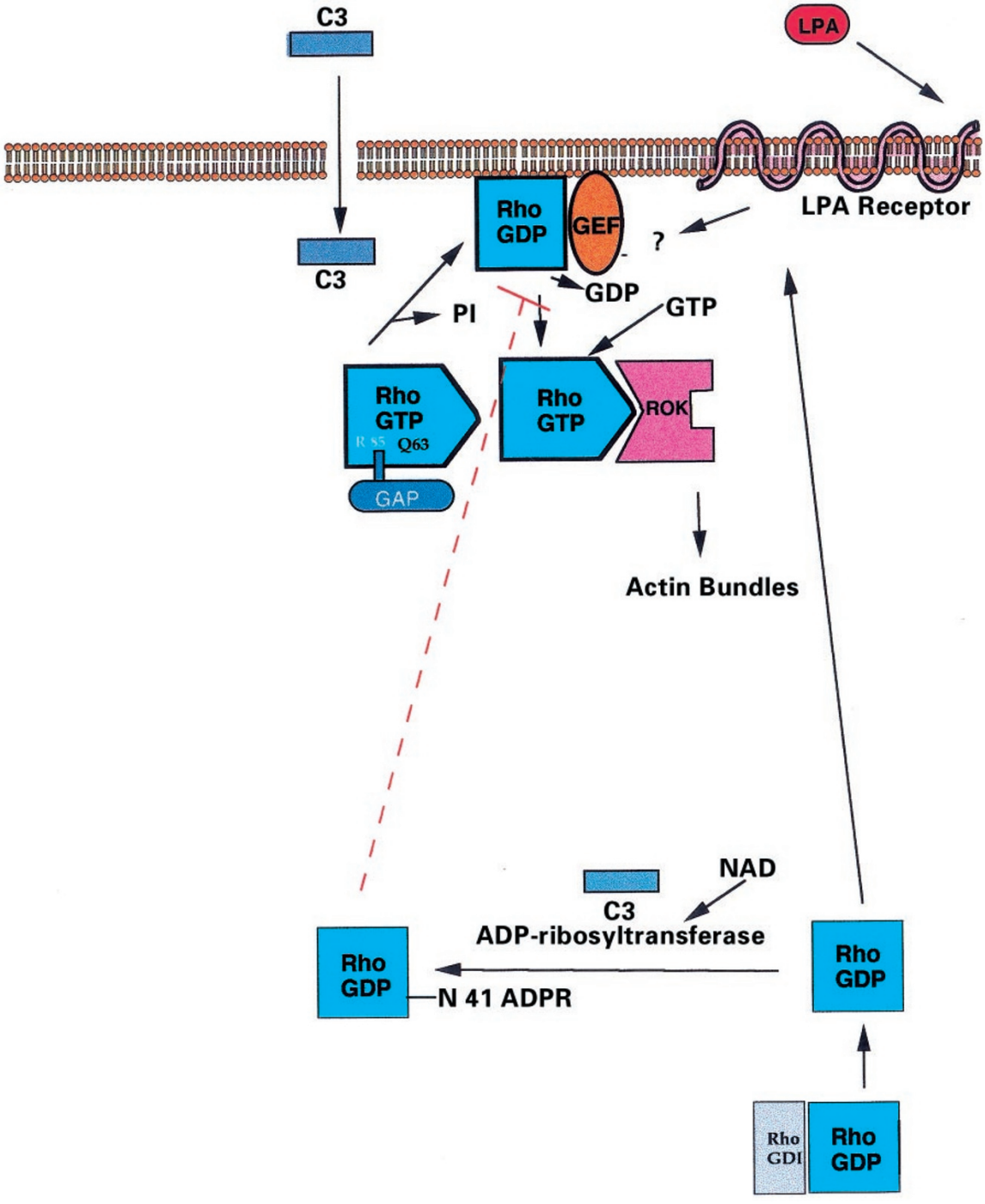

Figure 1 Activity of *Clostridium botulinum* exoenzyme C3 on Rho. C3 is not a toxin, thus it must be injected into the cytosol or introduced by chimeric toxins. Once in the cytosol C3 ADP-ribosylates Rho, preferentially the GDP bound form, on Rho asparagine 41. ADP-ribosylated Rho is unable to bind the cell membrane (red dotted line) thus it probably cannot be activated by the Rho GEF. However, *in vitro* ADP-ribosylated Rho can still bind the Rho downstream target ROK (black arrow)

by Rho through blood vessel twitch (Uehata *et al*, 1997). Another important phenomenon induced by Rho activity and demonstrated by utilization of C3 is the inhibitory role of Rho in an early step of receptor-mediated endocytosis (Lamaze *et al*, 1996).

The role of Rho in the regulation of gene transcription has been analyzed using C3. From these studies it seems that Rho can control transcription at the level of the promotor serum responsive element (SRE) via activation of the serum response factor (SRF) (Hill *et al*, 1995).

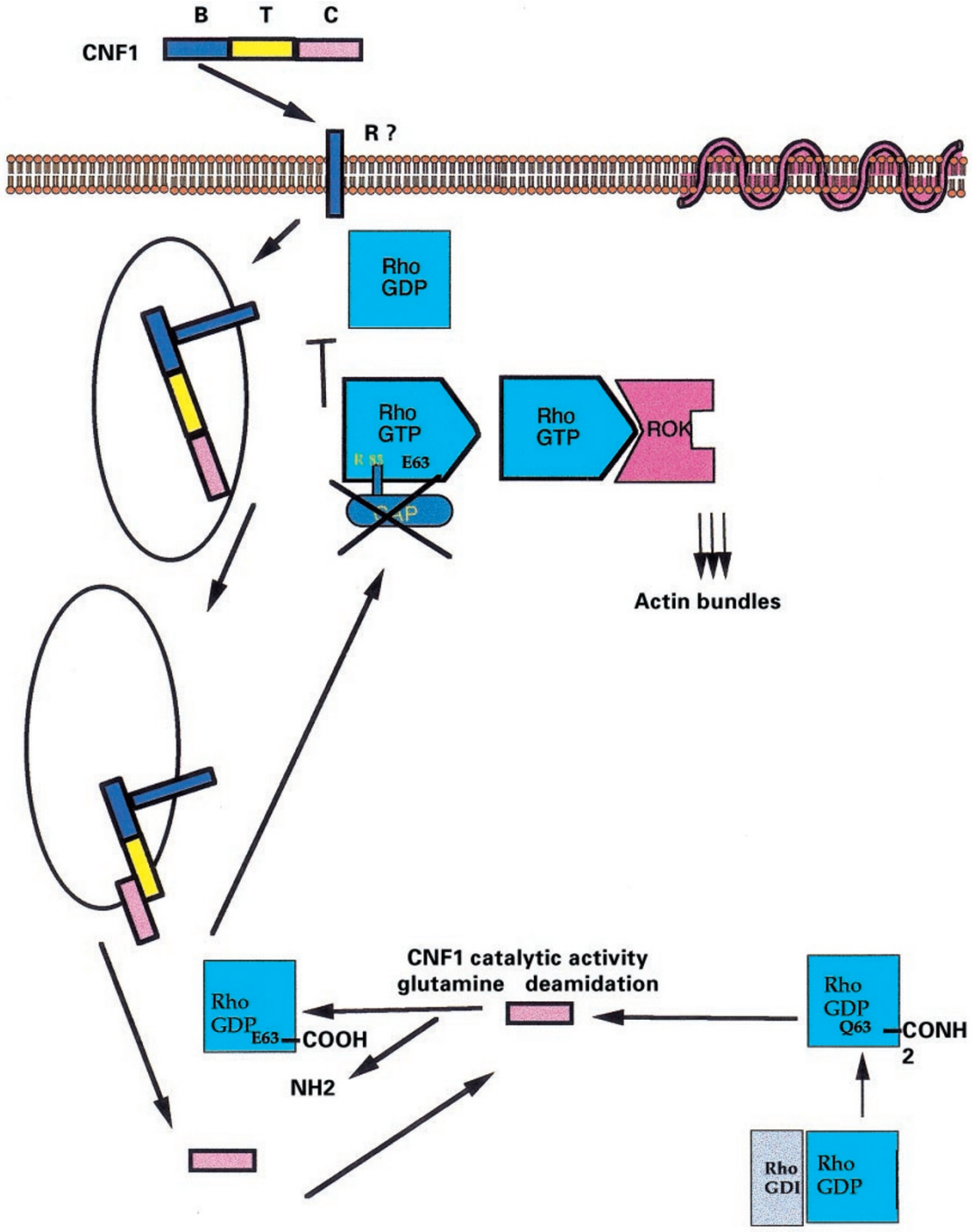

Figure 2 Activity of *Escherichia coli* CNF1 on Rho. CNF1 is organized into three domains B: cell binding, T: membrane translocation, C: catalytic. After binding to a membrane receptor, CNF1 is taken up by endocytosis and into an acidic intracellular compartment where the catalytic domain crosses the membrane, aided by the translocation domain. Once in the cytosol the catalytic CNF1 domain deamidates Rho glutamine 63 (Q63) into Rho glutamic acid 63 (E63). Rho E63 loses its ability to hydrolyse GTP into GDP in conjunction with RhoGAP. Rho being bound permanently to GTP activates the downstream Rho target ROK giving rise to stress fiber formation by microfilament bundling

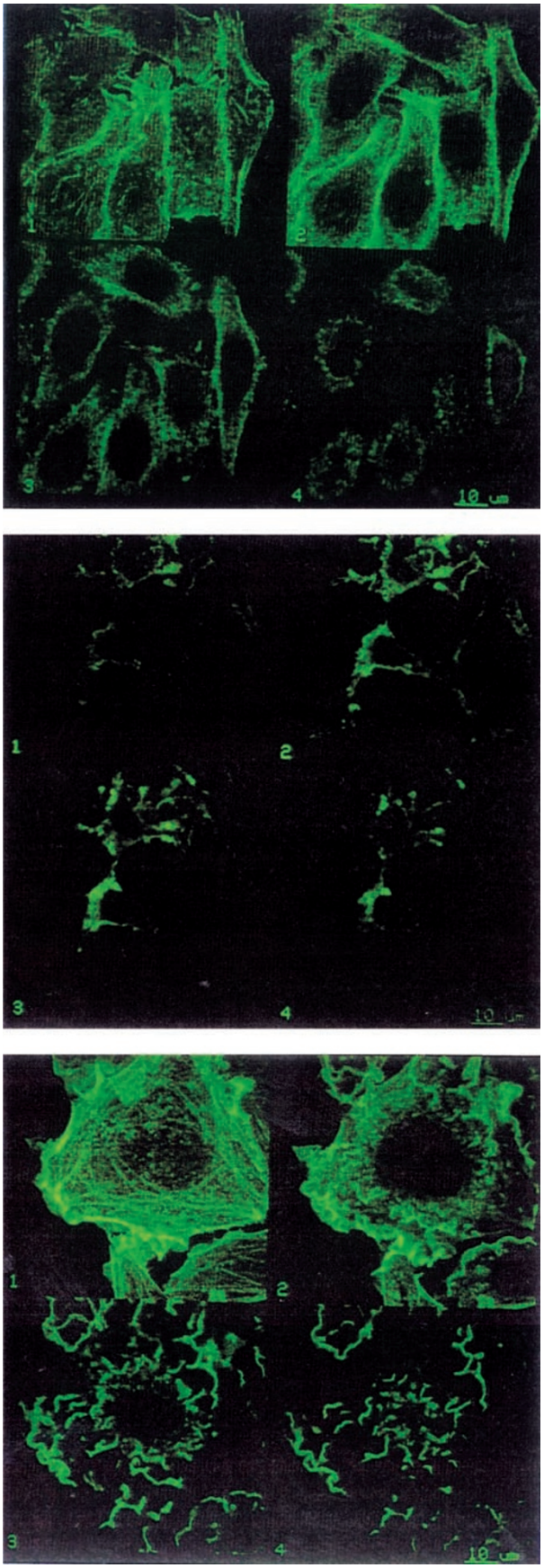

Table 1 Comparative cell activities of CNF1 and C3

Activity	**C3** (ref)	**CNF1** (ref)
Cell division	inhibits (Rubin *et al,* 1988)	activates (Lacerda *et al,* 1997)
Cytokinesis	inhibits (Rubin *et al,* 1988)	inhibits (Caprioli *et al,* 1983)
Stress fiber formation	inhibits (Chardin *et al,* 1989)	activates (Fiorentini *et al,* 1988)
Receptor-mediated endocytosis	activates (Lamaze *et al,* 1996)	?
Pinocytosis	inhibits (Schmalzing *et al,* 1996)	activates (Senda *et al,* 1997)
Contractility	inhibits (Fujihara *et al,* 1997)	activates (Fiorentini *et al,* 1997a)
Focal contact formation	inhibits (Aullo *et al,* 1993)	activates (Fiorentini *et al,* 1988)
PIP2 formation	inhibits (Chong *et al,* 1994)	activates (Fiorentini *et al,* 1997a)
Apoptosis	activates (Henning *et al,* 1997)	inhibits (Fiorentini *et al,* 1998)
Substrate	Rho (Chardin *et al,* 1997)	Rho (Flatau *et al,* 1997)
Co-substrate	NAD (Chardin *et al,* 1989)	none (Flatau *et al,* 1997)
Amino-acid modified	asparagine 41 (Sekine *et al,* 1989)	glutamine 63 (Flatau *et al,* 1997)
Enzymatic activity	ADP-ribosyltransferase (Rubin *et al,* 1988)	deamidase (Flatau *et al,* 1997)

However, activation of SRF by Rho does not depend on the MAP kinase or JNK/p38 pathways (Hill *et al*, 1995). Finally, C3 is able to block the entry of cells into G1 of the cell cycle indicating a role for Rho in the G0/G1 step (Olson *et al*, 1995).

CNF1 induces effects in cells opposite to those of C3 (Figure 3). It provokes a prominent bundling of actin stress fibers and multiplication of focal adhesion points (Fiorentini *et al*, 1988). This leads to cell spreading by the mechanism described above. CNF1 induces a large increase in enzymatic activity of the PI-4 5-kinase (Fiorentini *et al*, 1997a) but curiously it is not possible to detect in cells an elevation of the PIP2 concentration (Fiorentini *et al*, 1997a). CNF1 also induces phosphorylation of $p125^{FAK}$ kinase and paxillin which are localized in focal adhesion contacts (Lacerda *et al*, 1997) and provokes the relocalization of myosin type 2 into stress fibers (Fiorentini *et al*, 1997a). Recently, it has been shown that a toxin which exhibits an enzymatic activity identical to that of CNF1 on Rho (Horiguchi *et al*, 1997) stimulates the formation of caveolae (Senda *et al*, 1997), whereas C3 microinjection into *Xenopus* ovocytes blocked selectively the non-clathrin dependent endocytic pathway (Schmalzing *et al*, 1996). Caveolae, whose major protein is caveolin, are small

Figure 3 Confocal fluorescence micrographs of epithelial HEp-2 cells stained for F-actin. Four different focal plans (from the bottom (1) to the top (4) of the cell) are shown for each sample. (**A**) Control cells; (**B**) cells exposed to 10^{-9} M C3B (Aullo *et al*, 1993) for 18 h; (**C**) cells treated with 10^{-10} M CNF1 for 48 h. The main morphological effects provoked by these toxins in epithelial cells are: (**B**) cell retraction and actin cytoskeleton breakdown by C3, (**C**) cell spreading and actin assembly into ruffles and stress fibers by CNF1

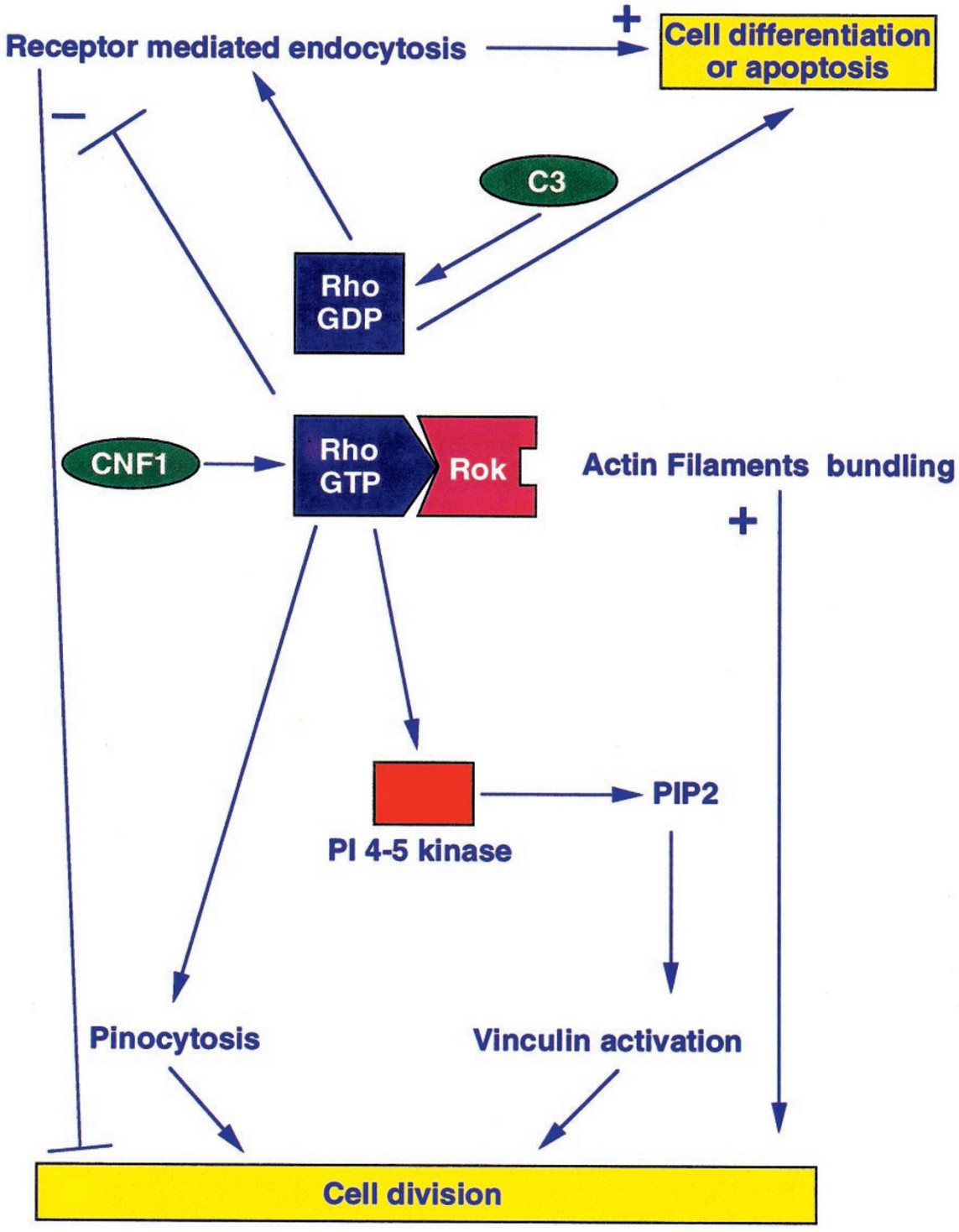

Figure 4 Possible pivotal role of Rho in cell multiplication, differentiation or apoptosis. Rho-GTP allows cell division by inhibiting receptor-mediated endocytosis and activating pinocytosis. This promotes filament bundling by both Rho kinase activation (filament bunding) and integrin cross-linking (vinculin activation) through PIP2 synthesis. Deactivation of Rho allows cell differentiation or apoptosis through microfilament disorganization, inhibition of pinocytosis and activation of receptor-mediated endocytosis. By permanently activating Rho, CNF1 provokes cell division and inhibits apoptosis. By inhibiting Rho, C3 blocks cell division but may allow either cell differentiation or apoptosis

plasma membrane invaginations, which can give rise to vesicle formation (Parton, 1996). It seems that caveolin can traffic between the plasma membrane and the trans-Golgi compartment (Conrad *et al*, 1995). It is quite well established that caveolae are privileged sites where growth factor receptors and molecules implicated in signalling are concentrated (Lisanti *et al*, 1994; Simons and Ikonen, 1997). Rho may thus play an important role in controlling the steady state between the clathrin coated vesicles and caveolin endocytic pathways. We have summarized in Table 1 the cellular activities modulated by C3 and CNF1.

Is Rho pivotal in driving cells toward multiplication, differentiation or apoptosis?

Cells which constitute an organism must imperatively attach to a substrate to multiply and differentiate (Ruoslahti and Reed, 1994). If conditions for attachment to the extracellular matrix are not suitable, the cell may induce its own suicide (Hynes, 1992). In vertebrates, cell adhesion is mainly due to integrins. There are many different integrins, each of them recognizing a specific extracellular matrix. Interactions between integrins and extracellular substrate may induce proliferation, diferentiation or apoptosis. There are therefore signalling cascades initiated from integrins and acting at the level of gene expresion. Integrins act in the signalling cascade by phosphorylation of protein tyrosine residues (Clark and Brugge, 1995), allowing the binding of molecules called adaptors (Pawson, 1995). This allows the binding of other proteins and initiates a chain of signalling reactions, the best known being the one linking Ras to MAP kinases implicated in cell mitogenicity (Wittinghofer and Nassar, 1996).

In order to explain the role played by Rho in cell regulation we took into account observations on Rho-dependent cellular effects reported by different groups using toxins like C3 or CNF1. First, the group of Sandra Schmidt has shown that Rho, bound to GTP, inhibits the formation of clathrin coats on the membrane, thus blocking endocytosis of cell receptors such as transferrin receptors (Lamaze *et al*, 1996). The same group has demonstrated an essential role for endocytosis mediated by growth factor receptors, such as EGF receptors, in the activation of these receptors (Viera *et al*, 1996). To summarize briefly, the inhibition of EGF receptor internalization stimulates their cell mitogenic activity, and increases the phosphorylation of the molecule called shc which can recruit adaptor proteins (Viera *et al*, 1996). On the other hand, internalization by clathrin coated vesicles of these receptors increases their ability to activate the PI3 kinase (Viera *et al*, 1996). This observation may be linked to that made by the group of Filippo Giancotti who reported that certain integrins induce cellular multiplication by provoking, through phosphorylation of tyrosine residues, the activation of the shc molecule (Wary *et al*, 1996). Shc then activates, by the Ras pathway, the MAP kinase cascade thus activating cell division (Wary *et al*, 1996). Curiously, however, shc in the Giancotti experiments was immunoprecipitated together with caveolin (Wary *et al*, 1996). The existence of caveolin in the induction of shc phosphorylation by integrins is explained by recruitment of both shc and a kinase by caveolae at the level of focal adhesion points (Wary *et al*, 1996). As discussed above, once activated by a CNF1-like toxin, Rho seems to increase the number of caveolae at the level of cell surface (Senda *et al*, 1997). Is it possible that Rho, by modulating the efficiency of endocytotic pathways together with its ability to cluster integrins, plays a central role in the decision of a cell to proliferate, differentiate or commit suicide? (Figure 4).

Thus, we propose the following scenario: a signal induced by an extracellular factor (for instance, LPA) will activate the receptor linked to Rho. Rho will induce in turn both fasciculation of actin stress fibers through activation of myosin (Chrzanowska-Wodnicka and Burridge, 1996) and the binding of actin filament to vinculin, by activation of this molecule with PIP2 (Gilmore and Burridge, 1996) thus allowing clustering of integrins (Chrzanowska-Wodnicka and Burridge, 1996). This mechanism will ensure the activity for signalling on an extracellular matrix. Concomitantly activated-Rho will increase the traffic and synthesis of caveolae (Senda *et al*, 1997) bringing shc and tyrosine kinases to the level of integrins (Wary *et al*, 1996). Moreover, it will inhibit the formation of clathrin coated vesicles (Lamaze *et al*, 1996) forcing receptors implicated in the control of mitogenicity to remain at the cell surface thus increasing their capacity to stimulate cell division (Viera *et al*, 1996).

After the arrest of the activating Rho signal, the Rho cascade will stop and the integrins will be dispersed by myosin light chain dephosphorylation and by the unbinding of vinculin on actin filaments. A new signal (required for differentiation) activating a regulatory cascade different from that of Rho will provoke the binding and clustering of a new set of integrins and the binding to a new extracellular matrix. Once Rho is deactivated, the endocytotic pathway mediated by clathrin will regain its activity and the receptors implicated in the mitogenicity will be inhibited by their own endocytosis. However, activation of the PI3 kinase by receptors, due to endocytosis (Viera *et al*, 1996), might stimulate new cascades of signalling such as those of Rac and Cdc42. This will be the case, for instance, if the new set of integrins recognize an extracellular matrix required for cell differentiation. On the other hand, if this extracellular matrix was not produced, the cascade of p38 kinases ending in apoptosis will be triggered by Rac or Cdc42 (Xia *et al*, 1995) to avoid a phenotypic error or an abnormal cellular multiplication. According to our model, activation of Rho will protect against apoptosis whereas inhibition of this GTP-binding protein, together with certain stimuli, will result in cell death. In agreement with this model, it has been shown that inhibition of Rho either by C3 (Bobak *et al*, 1997) or by *Clostridium difficile* toxin B (Gomez *et al*, 1997) is able to induce apoptosis in different cell types, whereas CNF1, by activating Rho, increases Bcl-2 expression and strongly protects against UVB induced apoptosis (Fiorentini *et al*, 1997b, 1998). Accordingly, when the exoenzyme C3 gene was expressed under the control of a T cell differentiation promotor, thymic cells from transgenic mice underwent apoptosis (Henning *et al*, 1997). In contrast with this view, it has been reported, for

example, that transfection of the dominant active version of Rho (Rho val 14) into 3T3 cells growing in the absence of serum was able to induce apoptosis (Jimenez *et al*, 1995). Further studies should be undertaken to solve this controversy.

A mitogenic signal must induce strong and constant phosphorylation to induce proliferation. The double signal from stimulated integrins and growth factor receptors activating the MAP kinase pathway, will explain the strong and sustained phosphorylation required for the entry of cells into the cell cycle. In our model, Rho might have no activating role *per se* on transcription, as demonstrated for Ras, Rac and Cdc42, but could play a role by coordinating the actin cytoskeletal organization together with the endocytic traffic in order to modulate the activity of certain kinases. In keeping with this model it seems that Rho exerts a permissive but not decisive effect on MAP kinase activation (Frost *et al*, 1996).

Conclusions

Bacterial toxins acting on the GTP-binding protein Rho, such as C3 and CNF1, are invaluable tools to study the functions of this regulatory protein whose pivotal role in cell homeostasis is becoming more and more evident.

The discovery that a bacterial toxin such as CNF1 (produced by pathogenic bacteria found in human and animal infections) induces an amino-acid modification which activates a small GTP-binding protein of the Ras superfamily leads us to pose the following provoking question: might certain tumors have an infectious origin?

Acknowledgements

This work was partially supported by National Research Council (CNR)-Strategic Project 'Cell cycle and apoptosis', U.O. 11, grant no. 97.04906.ST74 to C.F. and by funds from INSERM to P.B.

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