

# *Clostridium Botulinum* C3 Exoenzyme: Rho-Inactivating Tool in Cell Biology and a Neurotrophic Agent

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**Abstract:** C3 exoenzyme from *Clostridium botulinum* is the prototype of bacterial ADP-ribosyltransferases, which selectively modifies the Rho isoforms RhoA, RhoB and RhoC by covalent attachment of an ADP-ribose moiety. ADP-ribosylation results in inactivation of cellular functions of Rho. Because of its highly restricted substrate specificity, C3 is an established tool in cell biology; to this end C3 is applied as a cell-permeable chimeric toxin. C3 is superior to other molecular biology techniques such as siRNA or knock down approaches as RhoA inactivation or knock down is intrinsically associated with RhoB activation except after C3 treatment. RhoA plays an essential role in axonal growth and repair after neuronal injury. For therapeutic purposes cell-permeable C3 is now locally administered to treat spinal cord injury. Recently, it was reported that ADP-ribosyltransferase activity is not essential for the neurotrophic effect of C3 and that a peptidic fragment of C3 acts neurotrophic.

**Keywords:** ADP-ribosylation, Rho, Glucosylation, Neurotrophic effect.

## INTRODUCTION

C3 is produced by *C. botulinum* but is not related to the classical neurotoxins. C3 is an exoenzyme devoid of any attributable transport domain that is released by bacteria. Such domains are well-known delivery domains of bacterial protein toxins like cholera or pertussis toxin. The delivery domain consists of a receptor binding and translocation region that deliver the enzymic domain into the cytoplasmic compartment. Based on structural comparison, C3 is a mere enzyme but not a classical protein toxin. Nevertheless it reaches the cytoplasm of target cells by non specific uptake.

## ADP-RIBOSYLATION OF CELLULAR SUBSTRATES

C3 catalyzes mono-ADP-ribosylation of the low molecular weight GTP-binding proteins RhoA, RhoB, and RhoC [1,2]. The ADP-ribose moiety from ubiquitous cellular NAD<sup>+</sup> is N-glycosidically attached to the acceptor amino acid Asn-41 [3]. Thereby, two negatively charged phosphate groups are incorporated into the Rho protein resulting in its functional inactivation [4-6].

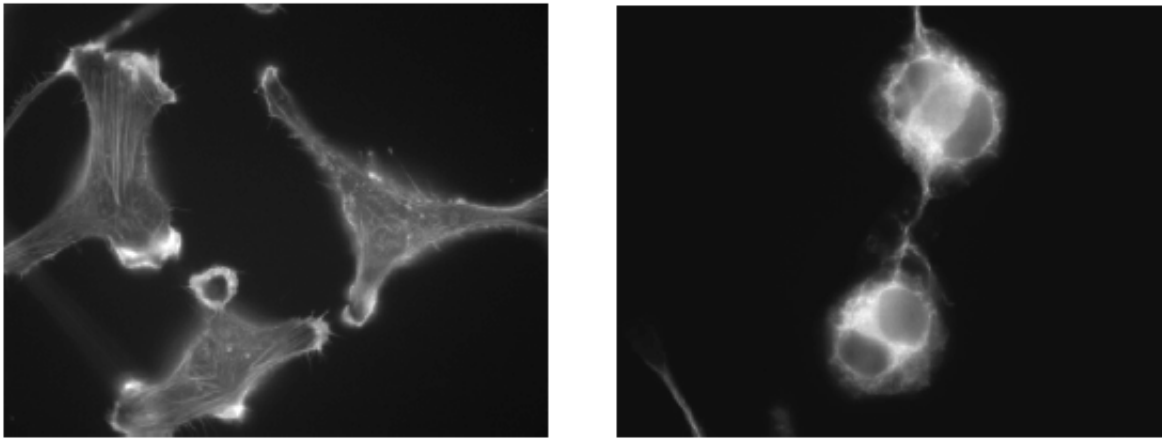
The substrate protein RhoA belongs to the Rho family of Ras proteins. They are guanine nucleotide-regulated monomeric GTP-binding proteins, which drive specific intracellular signalling. The Rho family is composed of several subfamilies: Rho(A,B,C), Rac(1,2,3)/RhoG, Cdc42/-TC10/TCL, Rnd(1,2,3), RhoH, RhoD/F. Only the highly homologous RhoA, RhoB and RhoC are substrates for C3 but not the other members. Rho, Rac, and Cdc42 are the master regulator of the actin cytoskeleton and cytoskeleton-associated functions such as cell shape, motility, phagocytosis, and smooth muscle contraction. RhoA regulates

formation of actin stress fibres, while Rac1 and Cdc42 control lamellipodia and filopodia formation, respectively. In addition, Rho is involved in the activation of transcription factors, cell cycle progression, cell transformation, and contractile ring formation in cytokinesis. While RhoA and RhoC are constitutively expressed, RhoB is a short-lived immediate-early gene product, which is up-regulated in response to cellular and genotoxic stress. RhoB (as well as RhoC) contributes to vesicle trafficking and cancer development [7-10].

ADP-ribosylation renders Rho incapable of down-stream signalling. Competence of down-stream signalling is governed by guanine nucleotides. Inactive, signalling-incompetent RhoA is bound to GDP and localized in the cytoplasm as a complex with GDI-1 (guanine nucleotide dissociation inhibitor-1), which stabilizes the inactive state. Activation results in release of RhoA from GDI-1 complex, translocation to the membranes and loading with GTP. Binding to GTP induces a conformational change (in switch I and II), which allows active RhoA to interact with and activate effector proteins [11]. Such proteins are kinases, lipases and scaffold proteins, which execute and amplify the Rho signal [12]. For example, activation of effector Rho-kinase (ROCK) by GTP-bound RhoA results in phosphorylation of myosin to increase F-actin contractility [7,8,13].

Asn-41, the acceptor amino acid of the ADP-ribose, resides in switch I, which is the effector binding region with which Rho binds to the effector protein. Interestingly, the bulky ADP-ribose does not block binding of RhoA to its effector proteins. Instead, the ADP-ribose blocks GTP loading catalyzed by the guanine nucleotide exchange factor (GEF). Furthermore, ADP-ribosylation prevents release of RhoA-GDP from the GDI-1 complex. Both sequestration to the GDI-1 complex and inhibition of GEF-induced GTP-loading, entraps RhoA in an inactive GDP-bound state and

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**Fig. (1).** C3-induced re-organization of the actin cytoskeleton. C3<sup>lim</sup> was applied to HeLa cells using electroporation. After 8 h of incubation, cells were fixed and the actin cytoskeleton was visualized by rhodamine-phalloidin staining. Left panel: control, right panel: treatment with C3.

subsequently inhibits Rho-dependent downstream signalling [6,14-18].

The most prominent finding of cells treated with C3 exoenzyme is the disappearance of actin stress fibres and cell rounding [2,4,19]. The actin cytoskeleton, which can be visualized as stress fibres, lamellipodia, and filopodia, is a dynamic equilibrium between polymerizing and depolymerizing impact. Actin polymerization is triggered (among others) by the Rho effector protein Rho-kinase (ROCK). ADP-ribosylation of RhoA causes inactivation of Rho-kinase and inhibition of F-actin polymerization. In C3-treated cells, the equilibrium between actin polymerizing and depolymerizing is altered and depolymerizing activity preponderates resulting in disappearance of stress fibres and altered morphology (Fig. 1). This example illustrates that GTP-binding protein-mediated signalling is not mono-directional, but rather a network in which several extra- and intra-cellular impacts are synchronized.

### THE FAMILY OF C3-LIKE ADP-RIBOSYLTRANSFERASES

C3 from *C. botulinum* (C3<sup>bot</sup>) was the first Rho-ADP-ribosylating bacterial exo-enzyme described and continues as family prototype of C3-like ADP-ribosyltransferases. This family further encompasses C3 isoforms from *Clostridium limosum* (C3<sup>lim</sup>) [20], *Bacillus cereus* (C3<sup>cer</sup>) [21,22], and *Staphylococcus aureus* (C3<sup>stau</sup>) [23] (Table I). The C3-like ADP-ribosyltransferases are secreted by means of a signal peptide and, thus, are classical bacterial exo-enzymes. The overall identity is about 35 % but all C3 isoforms harbour

highly conserved motifs: The ARTT motif (ADP-ribosylation toxin-turn-turn loop) is involved in Rho substrate recognition and harbours the catalytic glutamic acid residue, the PN (phosphate-nicotinamide) loop is involved in binding of co-substrate NAD<sup>+</sup>, and the STS-motif which together with conserved arginine residues form the catalytic cavity [24, 25].

The C3<sup>stau</sup> isoforms differ in their substrate specificity from C3<sup>bot</sup>, C3<sup>lim</sup> and C3<sup>cer</sup>. In addition to RhoA/B/C, C3<sup>stau</sup> ADP-ribosylates Rnd3 also known as RhoE [23]. Rnd3/RhoE is constitutively active based on the missing GTPase activity. It is a functional antagonist of RhoA. However, modification of RhoE is poor compared to RhoA. The functional relevance of RhoE ADP-ribosylation is not clear so far [26-28].

In addition to their transferase activities, C3 isoforms can hydrolyze NAD<sup>+</sup> into ADP-ribose and nicotinamide in the absence of the protein substrate Rho. However, free ADP-ribose is not covalently bound to Rho nor to Rnd3/RhoE. This NAD<sup>+</sup> hydrolyzing activity is poor compared to the ADP-ribosyltransferase activity and only detectable under artificial conditions, for which reasoning suggests that the hydrolyzing activity likely does not possess biological relevance [29].

### NON-MODIFIED TARGETS OF C3

C3 interacts also with a cellular target but does not enzymically ADP-ribosylate the target. Two C3 isoforms bind independently from their inherent transferase activity to

**Table 1.** Substrate Specificity of C3 Isoforms

	Substrates	Targets	Neurotrophic
<i>C. botulinum</i> C3 C3 <sup>bot</sup>	RhoA,B,C	Ral	+
<i>C. limosum</i> C3 C3 <sup>lim</sup>	RhoA,B,C	Ral	-
<i>B. cereus</i> C3 C3 <sup>cer</sup>	RhoA,B,C	-	-
<i>S. aureus</i> EDIN C3 <sup>stau</sup>	RhoA,B,C + Rnd3/RhoE	-	-

Ral. Ral is a low molecular weight GTP-binding protein that belongs to the Ras family. Ral is involved in transformation, migration, vesicle trafficking and stress response. In a cell-free system, C3<sup>bot</sup> and C3<sup>lim</sup> (but not C3<sup>cer</sup> and C3<sup>stau</sup>) and Ral form a high affinity complex ( $K_D \sim 60$  nM). Formation of the C3-Ral complex does not result in ADP-ribosylation of Ral. In contrast, the ADP-ribosyltransferase activity of C3 is blocked in the Ral complex. Vice versa, sequestration of Ral in the C3-complex blocks Ral-dependent downstream signalling. The recently determined X-ray crystallographic structure shows that C3 interacts with a region around switch II. Substrate recognition of Rho, however, is mediated via switch I. The interaction of C3 with switch II of Ral implicates that C3 acts like a GDI (guanine nucleotide dissociation inhibitor) to stabilize the inactive GDP-bound form of Ral to prevent GTP-exchange, i.e. activation. Thus, activation of Ral is inhibited by C3 through binding but not ADP-ribosylation. Whether these findings from cell-free systems are of biological relevance remains to be clarified [30,31].

### C3 AS A TOOL IN CELL BIOLOGY

C3 possesses a highly-restricted substrate specificity, as only RhoA/B/C out of approximately 150 low molecular weight GTP-binding proteins are ADP-ribosylated by C3. For this reason, C3 is classified as an excellent tool in cell biology to study the role of RhoA in cellular functions [6, 32, 33]. This notion is still true in the era of knock-out cells and siRNA. Generation of RhoA knock-out cells is not compatible with the critical role of RhoA in contractile ring formation in cytokinesis [34]. Furthermore, application of RhoA-siRNA is accompanied by massive RhoB expression and activation [35]. This unwanted side effect is based on the fact that activity of the *rhoB* promoter is suppressed by RhoA [35, 36]. RhoA inactivation is thus intrinsically associated with RhoB expression. Strong RhoB expression is also observed upon treatment of cells with C3 [35]. C3, however, completely ADP-ribosylates (i.e. inactivates) RhoB as well as completely inactivates RhoA [37]. Application of C3 thus represents the only approach to effectively inhibit RhoA without concomitant RhoB activation.

The application of C3 as a tool in cell biology is limited by its poor cell accessibility. C3 is deficient of a delivery domain to reach the cytoplasm of cells. C3 enters cell (likely by pinocytosis) upon the application of micromolar concentrations and long incubation times (up to 24 – 48 h). [19, 38-40].

The poor cell accessibility of C3 is overcome by microinjection (on the single cell level) [4, 41-45] or permeabilization techniques using either digitonin [46], streptolysin O [47] electro-permeabilization [48, 49], or scrape loading [50]. Electro-permeabilization of HeLa cells enables the delivery of C3 to complete cell populations and biochemical studies on RhoA-dependent effects [34, 51].

Furthermore, the C3 gene was introduced into eukaryotic cells by transient and stable transfection with plasmids or by viral infection. Transgenic mice have also been created to specifically express C3 exoenzyme in thymocytes or lenses [52-58].

To simplify C3 handling and subsequent studies, C3 chimeras were constructed. Such chimeras make use of the

delivery domains of other toxins such as diphtheria toxin, which mediates its own cell entry at low concentrations. C3<sup>bot</sup> has been fused to the binding and translocation subunits of diphtheria toxin [59], the binding domain of *Clostridium botulinum* C2 toxin [60-62], or the Tat peptide from HIV [63, 64], which are all able to permeate membranes. The application of chimeric-C3-toxins requires lower toxin concentrations (compared to the mere exoenzyme) on cell lines sensitive to either diphtheria toxin, C2 toxin or the tat peptide. C3 is then taken up by an identified process and the C3 activity occurs upon hours of treatment. However, the interpretation of findings is not as simple as initially thought because at least C3<sup>bot</sup> and C3<sup>lim</sup> inhibit Ral signaling. Novel findings show that C3<sup>bot</sup> exhibits an enzyme-independent effect, at least upon neurons.

### THERAPEUTIC FEATURES

Rho proteins are pivotal players in axonal growth and repair after neuronal injury that include the spinal cord [65-69]. Injury leads to the scenario that axons make contact with growth inhibitory molecules such as myelin-associated glycoprotein (MAG) or Nogo. Thereby, via membrane receptors such as p75<sup>NTR</sup>, NgR, Lingo, distinct signalling pathways are activated whose common player is RhoA. RhoA is activated through these pathways, which causes activation of Rho kinase (ROCK). Active ROCK in turn leads to growth cone collapse (at the axon tip of the or damage site), i.e. inhibition of axonal growth and blocked repair processes [70-73]. Rho inhibition may thus represent a strategy to de-block growth arrest. In fact, Rho inhibition by C3 de-blocks and allows re-growth plus repair of axons in animal models [71]. Under the assumption that C3 must enter its target cells, C3 fused to cell-permeable peptides has been applied in these studies. The findings with different animal models have been so convincing in enhancing recovery that cell-permeable C3 (BA-210) is now tested in phase II of clinical studies to treat human spinal cord injury [74].

In the meantime, we found that enzyme-deficient C3 induces axonal growth, excluding that ADP-ribosylation of Rho is necessary for growth promotion [75]. More recently, we found that a 29 amino acid peptide fragment of C3 is sufficient for growth promotion [76]. While enzymically-active C3 affects neurons as well as glial cells, the C3 peptide does not act on glial cells. This is a remarkable finding, as glial cells are thought to mediate neural inflammation and formation of glial scars in response to injury [77]. The neurotrophic effect of the C3 peptide remains uncharacterized, in particular if it depends upon binding to a cell surface receptor or neuronal uptake of the peptide.

### IN SUMMARY

- C3 exoenzyme from *Clostridium botulinum* specifically ADP-ribosylates RhoA, RhoB, and RhoC from the Ras superfamily of low molecular weight GTP-binding proteins.
- ADP-ribosylation leads to functional inactivation of cellular Rho.

- Because of its high substrate specificity, Rho is applied as a tool in cell biological research. Cell permeable C3 constructs are used.
- Cell-permeable C3 is applied as a drug to treat spinal cord injuries.

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