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Rho-modifying C3-like ADP-ribosyltransferases

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Abstract C3-like exoenzymes comprise a family of seven bacterial ADP-ribosyltransferases, which selectively modify RhoA, B, and C at asparagine-41. Crystal structures of C3 exoenzymes are available, allowing novel insights into the structure-function relationships of these exoenzymes. Because ADP-ribosylation specifically inhibits the biological functions of the low-molecular mass GTPases, C3 exoenzymes are established pharmacological tools to study the cellular functions of Rho GTPases. Recent studies, however, indicate that the functional consequences of C3-induced ADP-ribosylation are more complex than previously suggested. In the present review the basic properties of C3 exoenzymes are briefly summarized and new findings are reviewed.

Introduction

Many bacterial ADP-ribosyltransferases are potent bacterial protein toxins and important virulence factors. After cellular uptake caused by highly efficient cell entry mechanisms, they modify eukaryotic target proteins with great specificity and often grossly affect biological functions of their targets. These properties of the toxins are the reason for their use as cell biological and pharmacological tools (Aktories 2000). Particularly successful pharmacological tools are ADP-ribosyltransferases of the C3 family, which modify Rho GTPases. In the 1990s, C3 exoenzymes turned out to be very valuable experimental keys to understand the wide array of diverse regulatory functions of Rho GTPases. In hundreds of papers, C3 exoenzymes have been widely employed as cell biological tools to elucidate the cellular functions of Rho GTPases. This holds true despite the fact that these ADP-ribosyltransferases are rather poorly taken up by eukaryotic targets cells and their roles as virulence factors are still not well defined. Here, we will briefly review the basic properties of these ADP-ribosyltransferases and will focus on novel findings on the functional consequences of C3-induced ADP-ribosylation and discuss recent reports on the structure and function of the enzymes.

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Recent reviews about C3 exoenzymes focused on different aspects of the transferases (Aktories et al. 1992; Aktories 1997a,b; Boquet et al. 1998; Just et al. 2001; Narumiya and Morii 1993; Wilde and Aktories 2001). The exciting follow-up of the initial major discoveries in the field of Rho GTPases, including the role of C3 in this process, was recently vividly communicated by Ridley and Hall (Ridley and Hall 2004).

Sources of C3-like ADP-ribosyltransferases

C3 exoenzymes are produced by different types of Gram-positive obligate and facultative pathogens. So far, seven C3-like isoforms have been described, which are produced by *Clostridium botulinum*, *Clostridium limosum, Bacillus cereus* and *Staphylococcus aureus.* C3 was first identified as a product of *Clostridium botulinum* types C and D (Aktories et al. 1987, 1988; Rubin et al. 1988). Later it was found that two isoforms are produced by these Clostridia, which are about 65% identical in their amino acid sequences (Nemoto et al. 1991). They have been termed C3bot1 and C3bot2. At least the gene for C3bot1 is located on the same phage, which also encodes *C. botulinum* neurotoxins type C (Popoff et al. 1990). C3lim is produced by *Clostridium limosum* (Just et al. 1992) and is about 63% identical with C3bot1. *Bacillus cereus* produces C3cer (Just et al. 1995a), which is about 30% identical with C3bot1. Three C3 isoforms have been described, which are produced by *Staphylococcus aureus* (C3stau1, 2, and 3). These exoenzymes are about 35% identical with C3bot1 and 66%–77% identical between each other. The C3stau exoenzymes are also termed EDINs (Epidermal differentiation inhibitor) (Inoue et al. 1991; Wilde et al. 2001b; Yamaguchi et al. 2001) (Fig. 1a, b).

Structure–function analysis of C3-like exoenzymes

C3-like ADP-ribosyltransferases are enzymes of about 25 kDa, which all share the same activity in the sense that they mono-ADP-ribosylate RhoA, B, and C at the same site at asparagine 41 (Aktories et al. 1989; Braun et al. 1989; Chardin et al. 1989; Just et al. 1992, 1995a; Quilliam et al. 1989; Sekine et al. 1989; Sugai et al. 1992; Wilde et al. 2001b). The bacterial exoenzymes possess no receptor-binding or translocation domain and, consist exclusively of the catalytic domain, which possess ADP-ribosyltransferase and like many other ADP-ribosyltransferases also NAD glycohydrolase activity (Fig. 1a). Most important for understanding of the structure-function-relationship of C3-like transferases were the analysis of the crystal structures of C3bot either bound or unbound to NAD (Han et al. 2001; Ménétrey et al. 2002). These studies showed that the exoenzymes are very similar in structure and folding and share almost all functionally pivotal residues despite the limited primary sequence homology (some are not more than ~30% identical in their amino acid sequences). These data also corroborated previous mutational analysis, which let to the identification of many functionally important residues and their possible role in the ADP-ribosylation reaction (Böhmer et al. 1996; Just et al. 1995a; Saito et al. 1995; Wilde et al. 2002b).

The active site of C3bot (and most likely of other C3-like ADP-ribosyltransferases) consists of a mixed α/β-fold with a β-sandwich core, consisting of a five-stranded mixed β-sheet perpendicularly packed against a three-stranded antiparallel β-sheet. Four consecutive α-helices surround the three stranded β-sheet. An additional α-helix flanks the five-

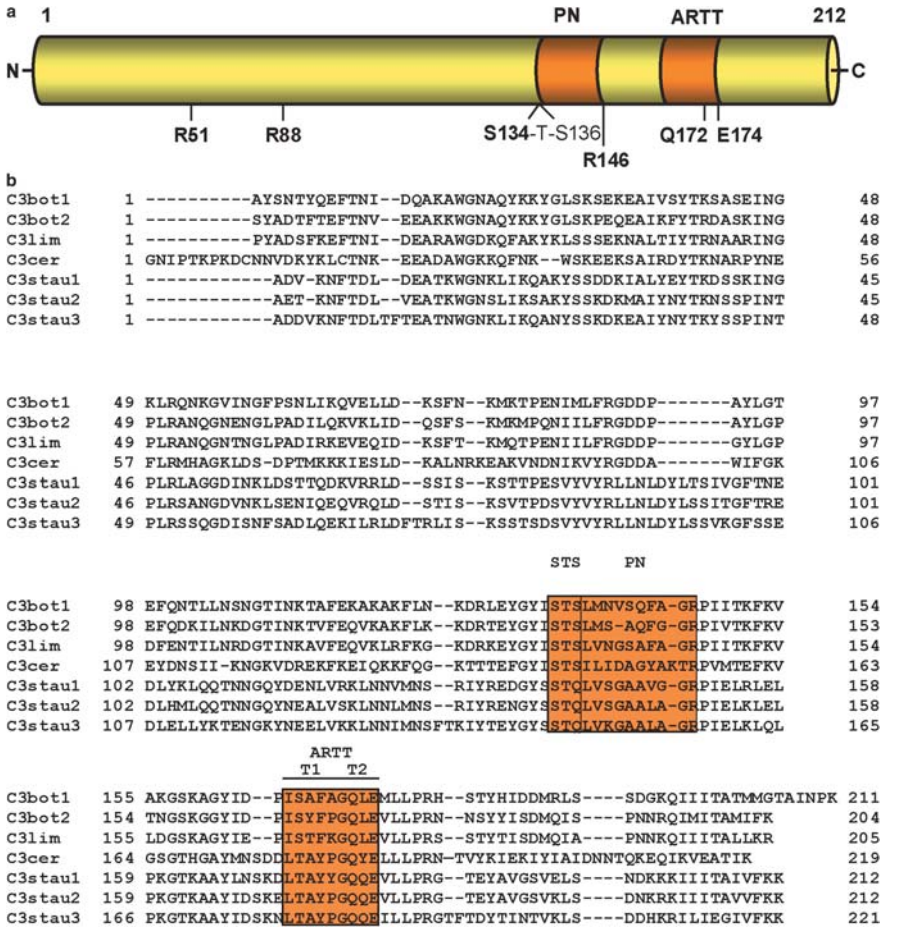
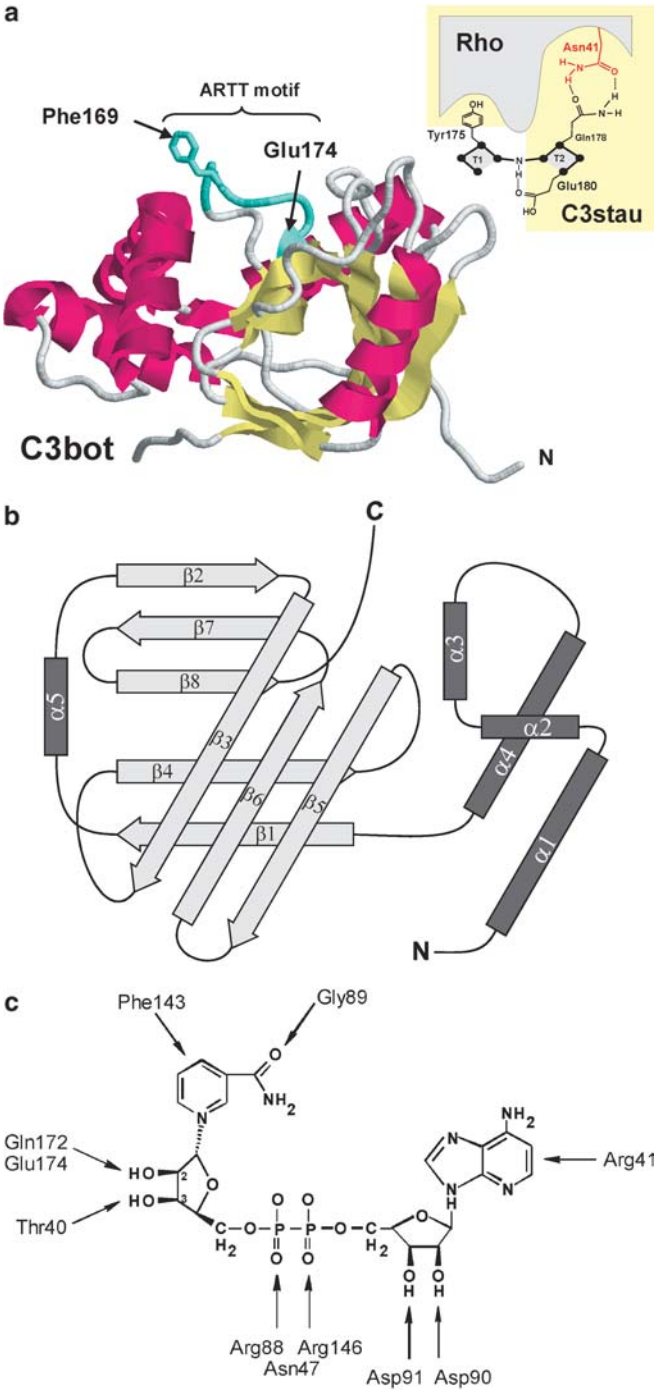


Fig. 1 Structure of C3 transferase. **a** Scheme of the primary sequence of C3bot showing the catalytic glutamate, residues of the ADP-ribosylation toxin-turn-turn (*ARTT)* loop, which is involved in protein substrate recognition, and PN-loop, which is involved in binding of phosphates of NAD. The STS-motif, which is conserved within the family of ADP-ribosyltransferases (C3stau isoforms possess an STQ motif), and several arginine residues involved in interaction with NAD are shown. **b** The sequences of the seven C3-like ADP-ribosyltransferases are given. *Clostridium botulinum* C3 transferase type I (C3bot1; Acc.Nr. P15879), *Clostridium botulinum* C3 transferase type II (C3bot2; Acc.Nr. Q00901), *Clostridium limosum* C3 transferase (C3lim; Acc.Nr. Q46134), *Bacillus cereus* C3 transferase (C3cer; Acc.Nr. AJ429241.1), *Staphylococcus aureus* C3 transferase A, B, and C (C3stau1; Acc.Nr P24121; C3stau2; Acc.Nr BAC22946, C3stau3; Acc.Nr;. NP_478345 ; also termed EDIN A,B,C).

stranded sheet (Han et al. 2001). After binding of NAD, a clasping movement ("Crab-claw" movement) of the transferase occurs which involves the structural elements α5, β2, β7 and β8, and α3 to enclose the substrate (Evans et al. 2003; Ménétrey et al. 2002) (Fig. 2). A novel structural motif, termed "ADP-ribosylating-toxin-turn-motif" (ARTT-motif) was proposed to be involved in the ADP-ribosylation reaction and suggested to be typical for all Rho-modifying C3-like transferases and also for the structurally related actin-modifying ADP-ribosyltransferases like *Clostridium botulinum* C2 toxin (Han and Tainer 2002). In C3bot, this motif consists of residues 167–170 (note that the counting is


a
Rho
Asn41
ARTT motif
Phe169
Glu174
Tyr175
Gln178
T1
T2
Glu180
C3stau
C3bot
N
b
C
β2
β7
β8
α5
α3
α2
β3
β4
α4
β6
β5
β1
α1
N
c
Phe143
Gly89
Gln172
Glu174
Thr40
Arg41
Arg88
Asn47
Arg146
Asp91
Asp90

without the signal sequence of 40 residues) for "turn 1" and residues 171–174 for "turn 2" (Figs. 1a, b, 2a). Turn 1 contains a conserved aromatic residue (C3bot^{Phe169}). The aromatic side chain points to the surface of the molecule and was suggested to recognize the substrate RhoA via hydrophobic patches around the acceptor amino acid residue Rho Asn41. The exchange of this critical residue to alanine or lysine in C3stau2 leads to a decreased binding of RhoA and abolishes the ADP-ribosyltransferase activity of these mutants (Wilde et al. 2002b). In the second turn, two residues (C3bot^{Gln172} and C3bot^{Glu174}) play important roles in enzyme activity. The side chain of C3bot^{Gln172} forms hydrogen bonds with the O2'-hydroxyl of the nicotinamide ribose (Fig. 1b), and is thought to be involved in the positioning of the ternary C3-NAD-Rho complex on turn 2. The side chain of C3bot^{Glu174} stabilizes the formation of an oxocarbenium transition state that arises during the enzymatic reaction (Han et al. 2001; Ménétrey et al. 2002; Oppenheimer 1994) (Fig. 2c). Exchange of either of these glutamine or glutamate residues to any other amino acids results in inhibition of the asparagine-modifying ADP-ribosyltransferase activity (Böhmer et al. 1996; Evans et al. 2003; Ménétrey et al. 2002; Saito et al. 1995; Wilde et al. 2002b).

Recently, it was reported that the ARTT-motif of C3bot undergoes conformational changes upon NAD-binding. While NAD is bound to C3bot, the complete motif is orientated into the inside of the protein and participates in NAD binding (Ménétrey et al. 2002). This form of NAD-binding was also observed in other ADP-ribosyltransferases (Bell and Eisenberg 1996; Choe et al. 1992; Han et al. 1999; Li et al. 1996). In C3stau2, the resting (NAD free) position of the ARTT loop is similar to the NAD bound state in C3bot. C3 exoenzymes produced by *S. aureus* are unique as compared to the other C3 transferases, because the loop before the ARTT loop possesses an additional two residues. These two residues are suggested to be responsible for positioning the ARTT loop of C3stau isoforms in a conformation identical not to that of the NAD-free C3bot1 structure but to that of the C3bot1-NAD-bound conformation. Therefore, conformational changes subsequent to NAD binding are minor in this region (Evans et al. 2003).

Deduced from the crystal structure of C3bot bound to NAD, a further structural element, termed phosphate-nicotinamide-loop (PN-loop), was suggested to be involved in NAD-binding (Fig. 1b). It covers residues C3bot 137–146 and is also located between strands β3–β4. At least one critical arginine within this loop is conserved in all C3-like ADP-ribosyltransferases. It was reported that this residue forms hydrogen bonds to the phosphate groups of NAD (Fig. 1b). Consequently, exchange to aspartate in C3bot or C3cer abolished both NAD glycohydrolase activity and ADP-ribosyltransferase activity of this mutant (Ménétrey et al. 2002; Wilde et al. 2003).

Enzyme activity and substrate specificity

Like typical ADP-ribosyltransferases, C3 exoenzymes split NAD into ADP-ribose and nicotinamide and transfer the ADP-ribose moiety onto Rho protein (Fig. 3). C3 modifies

Fig. 2 Structure of C3bot. **a** The crystal structure of C3bot shows the ADP-ribosylation toxin-turn-turn (*ARTT*) motif. This motif is suggested to be involved in the interaction with the protein substrate, e.g., RhoA (C3 was designed by Swiss-Pdb Viewer 3.7 (database code 1G24). The insert exhibits the putative interaction of C3stau with RhoA. Data are from Han et al. (Han et al. 2001). **b** Scheme of the folding of C3bot (see text). **c** Residues, which participate in the binding of NAD to C3bot. Note that the counting of residues is without the signal sequence.

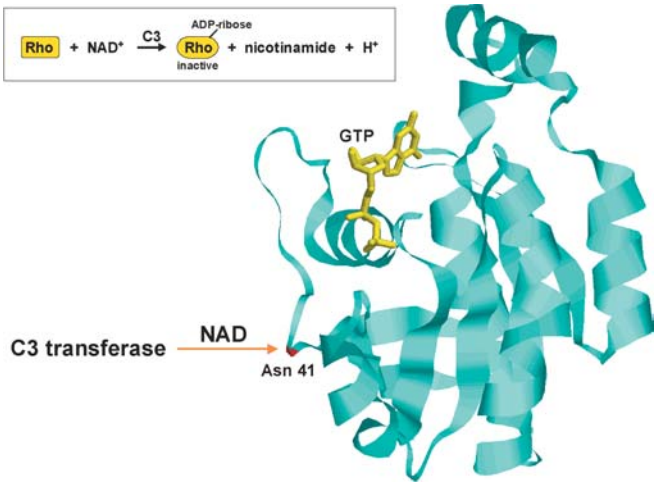


Fig. 3 ADP-Ribosylation of RhoA by C3. C3 transferases ADP-ribosylate RhoA at position Asn41. The acceptor amino acid is located in or very close to the so-called effector region (switch I-region) of RhoA. Rho structure was designed by Rasmol version 2.7.2 (database code 1FTN).

asparagine residue (Asn41) of the target protein (Sekine et al. 1989). This is unique for this family of ADP-ribosyltransferases. Many bacterial ADP-ribosyltransferases modify arginine residues, including cholera toxin, *Pseudomonas exoenzyme* S and T, and the actin modifying binary ADP-ribosyltransferases like *C. botulinum* C2 toxin. Cysteine is modified by pertussis toxin (Aktories 2000; Barbieri et al. 2002).

C3-like ADP-ribosyltransferases are characterized by their substrate specificity, because they modify preferentially Rho A, B, and C. Other Rho GTPases are poor substrates, including Rac and Cdc42. Recently, it was reported that the transferases C3stau1 and C3stau2 (EDIN A and EDIN B) from *S. aureus* ADP-ribosylate also RhoE and Rnd3. RhoE and Rnd3 are isoforms, identical except for a 15-residue N-terminal extension on Rnd3, that are antagonistic to RhoA (Guasch et al. 1998; Foster et al. 1996; Nobes et al. 1998; Riento et al. 2003). They bind GTP but lack GTPase activity. However, the kinetics of the modification of RhoE/Rnd3 is much more slower than that to modify RhoA (Wilde et al. 2001b).

The targets of C3 exoenzymes are molecular switches

RhoA, B, and C, the main targets of C3 exoenzymes, belong to the Rho subfamily of low molecular mass GTP-binding proteins, which comprises more than twenty related GTP-binding proteins, including RhoA, B, C, Rac1, 2, 3, Cdc42, RhoD, Rnd1, Rnd2 (RhoN), RhoE/Rnd3, RhoF (Rif), RhoG, RhoH (TTF) and TC10, TCL, Chp, and Wrch (Jaffe and Hall 2002; Nagata et al. 1998; Ridley 2000; Wennerberg and Der 2004). Most of them, e.g., the prototypes RhoA, B, C, Rac, and Cdc42 cycle between an activated GTP-bound form and an inactive GDP-bound form (Fig. 4). The exchange is tightly controlled by regulating proteins: (a) guanine nucleotide exchange factors (GEFs; more than 60 have been identified) which activate Rho by promoting the exchange of GDP to GTP, (b) GTPase-

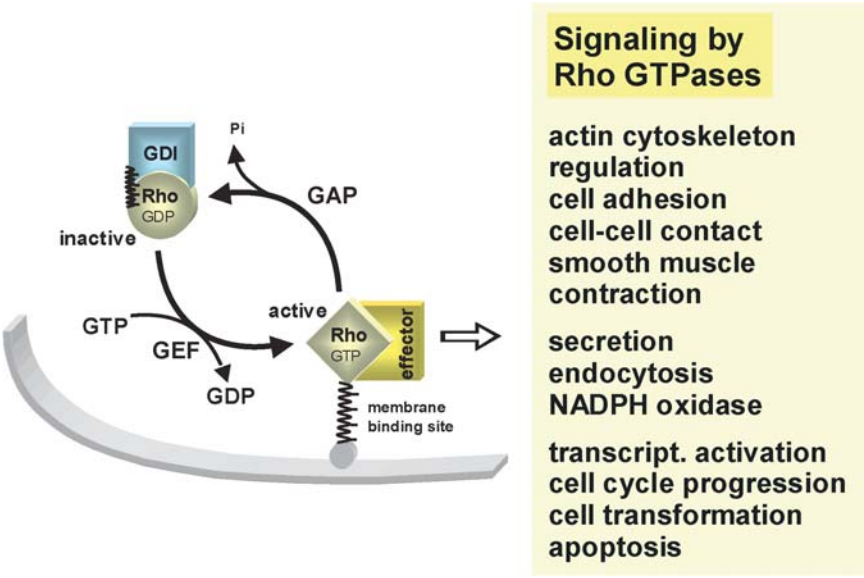


Fig. 4 RhoA GTPase cycle. Rho GTPases are inactive in the GDP bound form and activated by GDP/GTP exchange, which is facilitated by guanine nucleotide exchange factors (*GEFs*). In the active form RhoA interacts with a large array of effectors to induce various cellular effects indicated. The active form of RhoA is terminated by hydrolysis of the bound GTP, which is facilitated by GTPase-activating proteins (*GAPs*). The inactive form is extracted from the membrane by guanine nucleotide dissociation inhibitor (*GDI*), which keep the Rho GTPases in their inactive form in the cytosol.

activating proteins (GAPs; more than 70 have been identified), which inactivate Rho proteins by increasing their intrinsic rate of GTP-hydrolysis, and (c) guanine nucleotide dissociation inhibitors (GDIs; only three are known), which sequester the isoprenylated Rho proteins in the cytosol. The active GTP-bound form of Rho GTPases, which is mostly located at the cell membrane, interacts with multiple cellular effectors, including different protein kinases, lipid kinases, phospholipases and a still growing number of adaptor proteins, involved in a large array of distinct cellular functions, including regulation of the cytoskeleton (Burridge and Wennerberg 2004), cell and smooth muscle contraction, phagocytosis, polarity, activation of transcription, cell cycle progression, and cell transformation (Bishop and Hall 2000; Etienne-Manneville and Hall 2002; Jaffe and Hall 2002; Wennerberg and Der 2004).

Rho GTPases have been identified to be the preferred target of several other bacterial toxins and effectors. They can be activated by deamidation (*E. coli* cytotoxic necrotizing factors, CNF1, CNF2, and CNFy) (Flatau et al. 1997; Hoffmann et al. 2004; Schmidt et al. 1997) and by transglutamination (*Bordetella* dermonecrotizing toxin DNT) (Masuda et al. 2000) at Gln63/61 in Rho and Rac/Cdc42, respectively. Moreover, Rho GTPases are activated by *Salmonella* SopEs, which possess GEF activity and mimic the regulatory functions of endogenous activators (Hardt et al. 1998). An inactivation of Rho GTPases is caused by mono-O-glucosylation by the large clostridial cytotoxins, including toxins A and B of *Clostridium difficile* (Just et al. 1995b), lethal toxin from *Clostridium sordellii* (Just et al. 1996), and α-toxin from *Clostridium novyi* (Selzer et al. 1996). *Yersinia* YopE (von Pawel-Rammingen et al. 2000), *Salmonella* SptP (Fu and Galán 1999), or *Pseudomonas aeruginosa* ExoS (Goehring et al. 1999) inactivate Rho GTPases by mimicking en-

dogenous GAP activity. Moreover, it has been shown recently that *Yersinia* YopT acts as a protease, which cleaves Rho-GTPases at the C-terminal isoprenylated cysteine to inactivate the GTPase (Shao et al. 2002, 2003).

Functional consequences of the ADP-ribosylation of Rho

The ADP-ribosylation of RhoA (B, C) occurs at asparagine-41 (Sekine et al. 1989), which is part of or at least located in close vicinity to the switch-1 region (residues 28–40/41) of the GTPase (Fig. 3). The modification renders Rho biologically inactive (Paterson et al. 1990). The switch-1 region adopts different conformations depending on the nucleotide bound to the GTPase and is the molecular basis for the conduction of signals downstream. The inactivation of Rho by C3 exoenzyme-catalyzed ADP-ribosylation can be easily monitored by redistribution of actin filaments and depolymerization of stress fibers (Chardin et al. 1989; Paterson et al. 1990; Wiegers et al. 1991). ADP-ribosylation of Rho has only minor effects on the nucleotide binding, intrinsic, and GAP-stimulated GTP hydrolase activity. Also binding of ADP-ribosylated Rho with effector proteins, e.g., protein kinase N or Rho kinase (Sehr et al. 1998) and phospholipase D (Genth et al. 2003b) is possible (Fig. 5). Moreover, ADP-ribosylated RhoA is still able to activate its effectors (Genth et al. 2003a). However, this activation appears to depend on the fact that it is already in the active form before ADP-ribosylation. ADP-ribosylation appears to prevent the conformational changes occurring with activation of Rho proteins (Genth et al. 2003b). In line with this notion is the finding that activation of ADP-ribosylated Rho by GEFs (e.g., Lbc) is inhibited (Sehr et al. 1998) (Fig. 5b). Importantly, ADP-ribosylated RhoA seems to be trapped in the Rho/GDI-complex (Genth et al. 2003a). This was studied with a simple membrane filtration assay. The unmodified RhoA/GDI complex (mass ~45 kDa) is not able to pass a 30-kDa cut-off membrane filter. In the presence of phosphatidylinositol 4,5-bisphosphate (PIP2), the complex dissociates and releases RhoA (~20 kDa) and GDI (~24 kDa), which are able to pass the membrane filter. After ADP-ribosylation, however, PIP2 is not able to dissociate the RhoA/GDI-complex, indicating a tight interaction after modification of Asn41 by C3 (Fig. 5c). In line with the apparent increase in the affinity between modified RhoA and GDI, ADP-ribosylated RhoA is exclusively found in the cytosolic fraction of C3-treated cells. ADP-ribosylation reduces the binding of RhoA to membranes (Fujihara et al. 1997; Genth et al. 2003a). Taken together, inhibition of activation of ADP-ribosylated Rho by GEFs and sequestration of ADP-ribosylated RhoA in the GDI-complex are most likely the causes of C3-induced blockade of Rho-dependent signaling (Fig. 6).

Nonenzymatic actions of C3 exoenzymes

Recently, it was reported that C3-like exoenzymes interact directly with other small GTP-binding proteins not belonging to the Rho subfamily of GTPases. Wilde and coworkers showed that C3 exoenzymes from *C. botulinum* and *C. limosum* bind with high affinity (k_D ~10 nM, for *C. limosum*) to RalA (Wilde et al. 2002a) without modifying the GTPase by ADP-ribosylation. Ral is a member of the Ras subfamily of small GTPases and occurs in two isoforms, RalA and B, which share ~35% amino acid identity to RhoA. Ral has been implicated in several cellular processes, e.g., Ras-mediated cell transformation (Feig

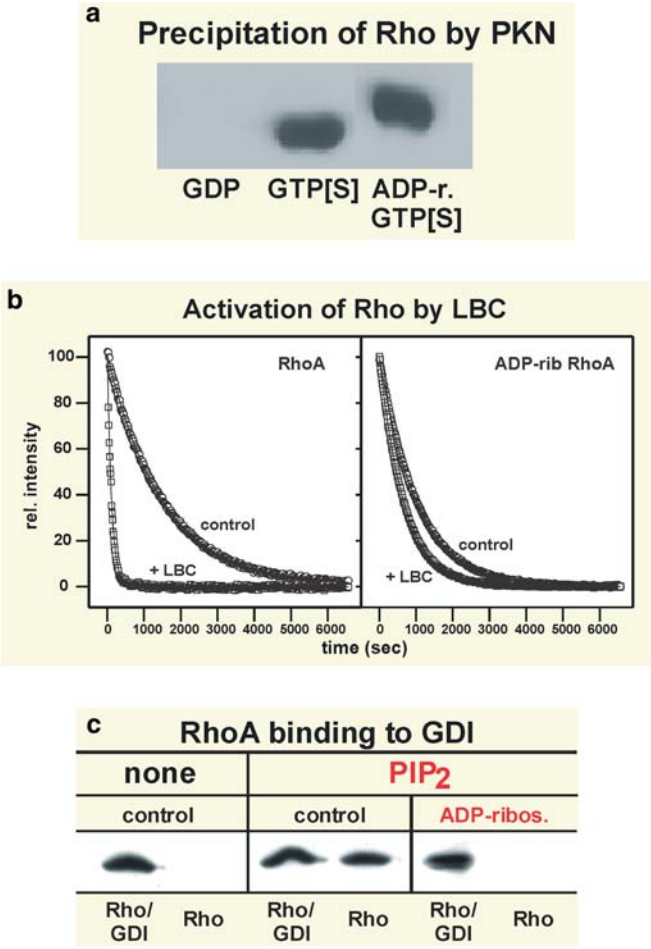


Fig. 5 Functional consequences of ADP-ribosylation of RhoA. **a** ADP-ribosylation of RhoA at Asn41 has no major effect on the interaction of the GTPase with the RhoA effector protein kinase N (*PKN*). Under control conditions, only the active GTP-form (here the GTPγS-form) but not the inactive GDP-form of RhoA is precipitated by the Rho-binding domain (*RBD*) of protein kinase N (*PKN*). In the experiment shown, this RBD-domain was coupled to Sepharose beads and used for precipitation. After ADP-ribosylation, which can be monitored by the shift of RhoA to an apparent higher molecular mass, RhoA is still able to interact with PKN (data from Sehr et al. 1998). **b** ADP-ribosylation decreases the rate of activation by the GEF protein LBC. The activation of RhoA was followed by the release of the fluorescently labeled mantGDP from RhoA to allow binding of GTP. Therefore, activation of RhoA causes decrease in fluorescence. Pretreatment of RhoA with C3 reduces the rate of RhoA activation (data from Sehr et al. 1998). **c** ADP-ribosylation increases the binding of RhoA to GDI. In the cytosol, Rho is in a complex with GDI. Therefore, only the complex is detected by gel or membrane filtration. Under control conditions phosphatidylinositol bisphosphate (*PIP2*) causes dissociation of this complex. Accordingly, Rho released from the complex is detected. After ADP-ribosylation PIP2 is not able to induce dissociation of the complex. Therefore, ADP-ribosylated Rho stays in the cytosol in a complex with GDI. (Data from Genth et al. 2003a).

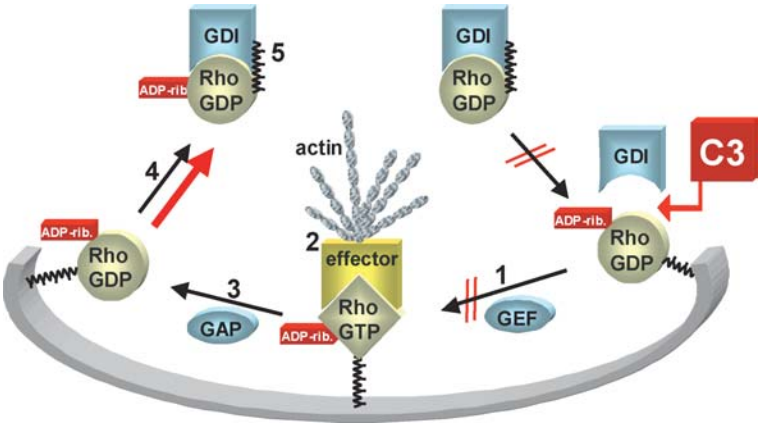


Fig. 6 Summary of the functional consequences of the ADP-ribosylation of RhoA by C3. RhoA is ADP-ribosylated by C3 in the GDI-free form. *1* ADP-Ribosylation inhibits the activation of RhoA by GEF. *2* ADP-ribosylated Rho is still able to interact at least with some effectors such as kinases. *3* The nucleotide binding and the GTP hydrolysis is almost not affected by ADP-ribosylation. *4* ADP-ribosylation decreases membrane-binding of RhoA. *5* Binding of ADP-ribosylated RhoA to GDI is increased. Therefore, ADP-ribosylated RhoA will remain in the inactive form in the cytosol.

et al. 1996; Urano et al. 1996), cytoskeleton rearrangement (Jullien-Flores et al. 1995; Ohta et al. 1999; Park and Weinberg 1995), and vesicle trafficking, e.g., by regulating the exocyst via binding to sec5 (Moskalenko et al. 2001). Ral acts on phospholipase D1 (PLD1) (Jiang et al. 1995; Luo et al. 1998) and it is suggested that both RalA and PLD1 modulate receptor endocytosis and vesicle transport. Binding of C3 to RalA inhibits its ADP-ribosyltransferase activity to modify RhoA (Fig. 7). Similarly, interaction of C3 with RalA reduces the ability of the GTPase to activate PLD1 in vitro, suggesting that the binding of the exoenzyme to Ral occurs in a region of the GTPase, which is important for the interaction with its effectors. Moreover, interaction of C3 with Ral prevents glucosylation of Ral by *Clostridium sordellii* lethal toxin in intact cells (Wilde et al. 2002a). Because glucosylation of Ral occurs in the functionally important switch-1 region, it is likely that interaction of C3 with this region also affects Ral functions in intact cells. Such a sequestration of Ral might be relevant at high concentration of C3, which can be achieved by microinjection or overexpression of C3 (see below). In contrast to the exoenzymes from *C. botulinum*, *C. limosum*, and *B. cereus*, the transferase C3stau2 from *S. aureus* is not capable of binding to RalA.

Recently another C3 effect, which is independent of the ADP-ribosyltransferase activity has been reported. It is well-known that Rho proteins regulate neurite outgrowth (see below). Several studies showed that C3 prevent neurite retraction induced by activated RhoA (see below). Surprisingly, Ahnert-Hilger and coworkers found that C3bot but not other C3 exoenzymes promote the axonal growth and branching independent of the enzyme activity (Ahnert-Hilger et al. 2004). Moreover, this effect depended on the extracellular application of the exoenzyme. Intracellularly expressed C3bot did not induce axon growth. They propose a novel neurotrophic function of C3bot independent of its transferase activity.

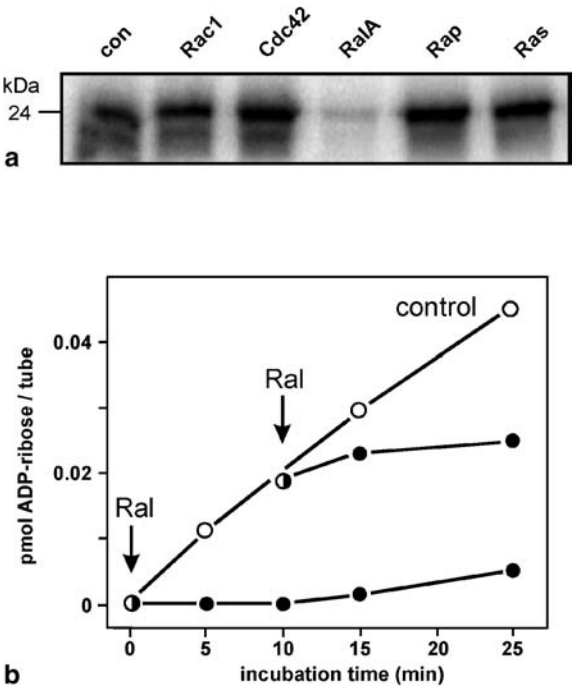


Fig. 7 Ral protein inhibits C3-induced ADP-ribosylation of RhoA. **a** RhoA was ADP-ribosylated by C3 with [^{32}P]NAD in the presence of various small GTPases (Rac, Cdc42, Ras, Rap, and Ral). Only Ral inhibited the ADP-ribosylation of RhoA. **b** Time course of ADP-ribosylation of RhoA in the presence and absence of Ral. Ral caused an immediate inhibition of the modification of RhoA. (Data from Wilde et al. 2002a).

Pathophysiological role of C3

Although much is known about the cellular functions of Rho GTPases, the roles of C3-like transferases in pathogenicity are not at all understood (Fig. 8). An action of C3 exoenzymes on the immune system of the eukaryotic target organism is most likely. Some of these effects have been already mentioned. Inhibition of immune cell functions including cytotoxicity of lymphocytes (Lang et al. 1992), adhesion (Nemoto et al. 1996), migration and invasion of lymphocytes (Stam et al. 1998; Verschueren et al. 1997), and leukocytes (Laudanna et al. 1996, 1997) by C3bot have been demonstrated. Rho GTPases have been proven to be important components of signal pathways used by antigen receptors, cytokine, and chemotaxins receptors to regulate the immune response (Heath and Holifield 1991; Henning and Cantrell 1998; Laudanna et al. 1996; Prepens et al. 1996; Price et al. 1995; Wojciak-Stothard et al. 1998). Moreover, Rho proteins participate in the barrier functions of epithelial cells (Nusrat et al. 1995; Vouret-Craviari et al. 1998) and in wound healing (Santos et al. 1997). However, considering the poor cell accessibility of C3 exoenzymes, an important question remains: how do these specific and potent agents get to their site of action? At least two possibilities are feasible. Recently, it was shown that pore-forming toxins appear to act as a delivery system for bacterial proteins. Madden and coworkers (Madden et al. 2001) reported that *Streptococcus pyogenes* uses streptolysin O, a cholesterol-dependent cytolysin, to translocate *S. pyogenes* NAD-glycohydrolase SPN into the target cells. This method of target-specific translocation appears to be comparable with the type-III secretion system frequently found in gram negative bacteria. Considering the fact that many of the bacteria, which synthesize C3 exoenzymes, also produce pore-forming agents, it is feasible that a similar mechanism is functional with these pathogens.

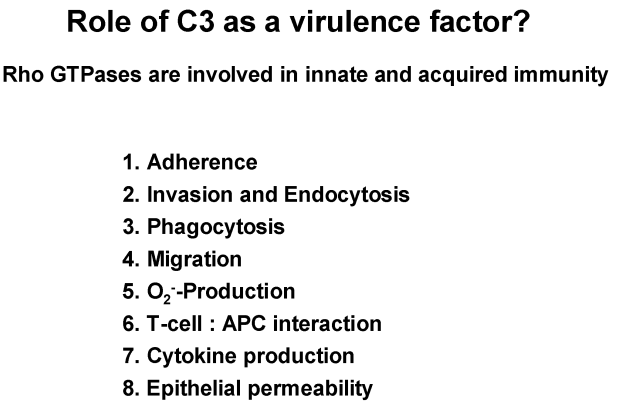


Fig. 8 The role of C3 as a virulence factor is not clear. However, Rho GTPases (including other members of the family) have been shown to be involved in several processes, which are important for innate and acquired immunity, including adhesion invasion and endocytosis of immune cells, migration, superoxide anion production (at least true for Rac), interaction of T-cells with antigen-presenting cells (*APC*), cytokine production, and epithelial permeability (see also Table 1).

The other possibility is based on recent findings that more pathogens than previously suggested are capable of invading host cells. This also applies to *Staphylococci* (Lowy 2000; Mempel et al. 2002). Moreover, it was suggested that the pathogens enter the cytosol of target cells (Bayles et al. 1998). This implies that the bacterium is able to release the Rho-ADP-ribosylating enzyme directly into the cytosol, where its protein target is localized (Wilde et al. 2001a; G.S. Chhatwal et al., unpublished data).

C3-like exoenzymes as pharmacological tools

Because C3-like ADP-ribosyltransferases are highly specific for Rho GTPases, they are established pharmacological and cell biological tools to study the physiological functions of Rho GTPases. On the other hand, C3-like ADP-ribosyltransferases lack a specific receptor binding and translocation domain and, therefore, their cellular uptake is rather poor. Due to this fact, the toxins have to be applied in high concentrations and/or for long incubation periods. Quite often the toxins were introduced into target cells by microinjection (Paterson et al. 1990; Watanabe et al. 1997). Another approach to overcome this problem is the use of C3-toxin chimeras. Aullo et al. (Aullo et al. 1993) fused C3bot to diphtheria toxin. DC3B, a fusion protein of C3 and the binding and translocation domain of diphtheria toxin, has a high affinity for the DT receptor, but apparently enters the target cell by a mechanism different from the typical pathway of diphtheria toxin. Because the action of this fusion toxin is limited to cells with receptors for diphtheria toxin, other chimeras were constructed. Very efficient is a fusion toxin, which is based on the binary C2 toxin from *Clostridium botulinum.* C2 toxin consists of the actin-ADP-ribosylating enzyme component C2I and the binding and translocation component C2II, which are both separated proteins (Aktories et al. 1986; Barth et al. 2002; Ohishi et al. 1980). After proteolytic activation of C2II, the activated C2II monomers oligomerize to heptamers (Barth et al. 2000) and upon binding of C2I to C2II, both components are internalized by receptor-mediated endocytosis. The N-terminal part (C2IN) of C2I, which alone is sufficient for the interaction with the binding component C2II, was fused to full-length ADP-ribosyltransferases C3lim or C3stau, respectively (Barth et al. 1998; Wilde et al. 2001b). This chimeric toxin increases the potency of C3 several hundred-fold (Meyer et al. 2000; Valderrama et al. 2000; Vischer et al. 2000; Wahl et al. 2000). Because the binding component of C2 toxin

appears to bind to complex and/or hybrid carbohydrates present on all vertebrate cells (Eckhardt et al. 2000), all these cells are sensitive towards the fusion toxin. Also the adaptor domain of the enzyme component and the binding component of iota toxin, which are similar to C2 toxin, have been effectively used for delivery of C3-like toxins into cells (Marvaud et al. 2002).

Recently, it was reported that C3bot could be transported into cells by adding short peptides to the C-terminal end of the exoenzyme. For this purpose short sequence of the human immunodeficiency virus transcription activator Tat was used (Park et al. 2003; Sauzeau et al. 2001). The transport of C3bot into cells can also be accomplished by fusing the third helix of the *Antennapedia* homeodomain protein from *Drosophila* to C3bot. In addition, short proline-rich peptides and highly basic arginine-rich peptides were C-terminally fused to C3 exoenzyme to facilitate the uptake of the transferase (Winton et al. 2002).

Another method to use C3-specific inhibition of Rho GTPases is the intracellular expression of the gene (Hilal-Dandan et al. 2004). Transgenes based on the thymocyte-specific lck promoter have been used for expression of C3 in thymus. Transgenic mice showed maturational, proliferative, and cell survival defects during T-cell development (Henning et al. 1997). Recently, a transgenic mouse model expressing C3 exoenzyme in a lens-specific manner was utilized (Maddala et al. 2004). Under transcriptional control of the lens-specific alphaA-crystallin promoter mice, expressing the C3 exoenzyme transgene, exhibited selective ocular defects, including cataract and microphthalmia (Rao et al. 2002).

In the following paragraph, cell biological effects, which are observed with the "C3 tool," are briefly summarized. Quite early studies showed that treatment of Vero cells with C3bot induces morphological changes characterized by rounding up of the cells with concomitant destruction of stress fibers (Chardin et al. 1989). The same findings were obtained with many other cell types and with different C3-like ADP-ribosyltransferases. Many of the classical studies on the functions of Rho GTPases performed in the laboratory of Alan Halls depended on the usage of C3 (Paterson et al. 1990; Ridley et al. 1992; Ridley and Hall 1992).

After C3 treatment, actin-staining by rhodamine-phalloidin usually reveals loss of stress fibers; treated cells remain in contact via small extensions. After removal of toxin from the medium, cells are still viable and the phenotype reverses after a few hours to days by neosynthesis of Rho (Barth et al. 1999). The reversal appears to be especially fast with the C3–C2I fusion toxin, which appears to be degraded rapidly (Barth et al. 1999). In many studies, C3 was shown to prevent the formation of stress fibers and focal adhesions induced by growth factor (Hall 1994; Mackay et al. 1997; Ridley and Hall 1992) or by integrins (Barry et al. 1997). In contrast, processes that are mediated by Rac or Cdc42, like lamellipodia and microspike formation in fibroblasts, are not affected by C3 (Kozma et al. 1995; Nobes and Hall 1995; Ridley and Hall 1992). Although C3bot induces rounding up in adherent cells, the toxins cause cell spreading in monocytes (Aepfelbacher et al. 1996) and in T cells (Borroto et al. 2000).

C3bot was frequently used as a tool to study the role of Rho in cell motility, migration and cell invasion (see Table 1). The exoenzyme was successfully applied in studies on the regulatory function of Rho GTPases in neurite outgrowth, branching, and neuroregeneration. Similarly the role of Rho GTPases in the control of phospholipase D and in phospholipid metabolism was studied with C3. The role of Rho GTPases in transcriptional activa-

Table 1 List of studies with C3 exoenzymes applied as cell biological and pharmacological tools

Rho in regulation of the actin cytoskeleton	Barry et al. 1997; Kozma et al. 1995; Nobes and Hall 1995; Ridley et al. 1992; Ridley and Hall 1992; Wiegers et al. 1991; Wilde et al. 2001b
Rho in cell adhesion, migration, chemotaxis and invasion	Adachi et al. 2001; Anderson et al. 2000; Kusama et al. 2001; Nguyen et al. 2002; Saurin et al. 2002; Strey et al. 2002; Takaishi et al. 1994; Worthylake et al. 2001; Yoshioka et al. 1998
Rho in cell cycle progression, cell division and cytokinesis	Eda et al. 2001; Fiorentini et al. 1998; Kato et al. 2001; Liberto et al. 2002; O'Connell et al. 1999; Olson et al. 1995; Takaishi et al. 1995
Rho and neurite growth	Dergham et al. 2002; Grunwald and Klein 2002; Jalink et al. 1994; Lehmann et al. 1999; Neumann et al. 2002; Niederost et al. 2002; Nishiki et al. 1990; Tigyi et al. 1996; Wahl et al. 2000; Yuan et al. 2003
Rho in endocytic and exocytic processes	Caron and Hall 1998; Doussau et al. 2000; Lamaze et al. 1996; Park et al. 2003; Schmalzing et al. 1995; Vögler et al. 1999
Rho, phospholipase D and phospholipid metabolism	Balboa and Insel 1995; Chong et al. 1994; Kanumilli et al. 2002; Kuribara et al. 1995; Meacci et al. 1999; Ren et al. 1996; Schmidt et al. 1996; Schmidt et al. 1999; Weernink et al. 2000; Xie et al. 2002
Rho and transcription and differentiation	Alberts et al. 1998; Chen et al. 2002; Dreikhausen et al. 2001; Hill et al. 1995; Lu et al. 2001; Mack et al. 2001; Sahai et al. 1998; Sotiropoulos et al. 1999; Su et al. 2001; Takemoto et al. 2002
Rho in signal transduction by heptahelical receptors	Buhl et al. 1995; Fromm et al. 1997; Gohla et al. 2000; Klages et al. 1999; Le Page et al. 2003; Mao et al. 1998a; 1998b; Nguyen et al. 2002; Ohmori et al. 2001; Sagi et al. 2001; Sah et al. 1996, 2000; Xie et al. 2002; Yamakawa et al. 2000
Rho and apoptosis	Bobak 1999; Dubreuil et al. 2003; Fiorentini et al. 1998; Hippenstiel et al. 2002; Mills et al. 1998; Reuveny et al. 2004

tion was another important topic, which was frequently addressed with C3 as a tool. Moreover, C3 was successfully employed in delineation of the role of Rho in the signaling of various heptahelical receptors to the actin cytoskeleton, phospholipases, and the nucleus via heterotrimeric G proteins. Especially important was C3 in studies on the functions of $G\alpha_{12/13.}$ Finally, C3 was studied in cell division and apoptosis (for references see Table 1).

Conclusion

Our information about C3 ADP-ribosyltransferases, their structures and mode of actions has increased enormously in recent years. We do understand a lot about the functional consequences of the ADP-ribosylation of Rho GTPases, when C3 is applied as a tool. However, additional potentially important functions and properties of C3 have been described recently, which are not clearly defined or not really understood at present, including the high affinity interaction with Ral and the action as a neurotrophic factor. Moreover, many open questions remain concerning the pathogenic role of C3 exoenzymes. With respect to further progress in the structure function analysis, it would be of major importance to solve the crystal structure of C3 in the complex with its Rho substrate. Hopefully, we will get this information in the near future.

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