REVIEW

C3 exoenzymes, novel insights into structure and action of Rho-ADP-ribosylating toxins

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Abstract The family of C3-like exoenzymes comprises
seven bacterial ADP-ribosyltransferases of different origin.
The common hallmark of these exoenzymes is the selective
N-ADP-ribosylation of the low molecular mass GTP-
binding proteins RhoA, B, and C and inhibition of signal
pathways controlled by Rho GTPases. Therefore, C3-like
exoenzymes were applied as pharmacological tools for
analyses of cellular functions of Rho protein in numerous
studies. Recent structural and functional analyses of C3-like
exoenzymes provide detailed information on the molecular
mechanisms and functional consequences of ADP-ribosy-
lation catalyzed by these toxins. More recently additional
non-enzymatic actions of C3-like ADP-ribosyltransferases
have been identified showing that C3 transferases from
Clostridium botulinum and *Clostridium limosum* form a
GDI-like complex with the Ras-like low molecular mass
GTPase Ral without ADP-ribosylation. These results add
novel information on the molecular mode of action(s) of
C3-like exoenzymes and are discussed in this review.

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Introduction

ADP-ribosylation is a biologically relevant protein modifi-
cation for physiological (e.g., DNA repair, intracellular
signaling, and extracellular regulation of immune cell
signaling) and pathophysiological processes since many
bacterial toxins were identified as mono-ADP-ribosyltrans-
ferases. After entry into target cells, which often involves
highly efficient uptake mechanisms, the toxins modify
eukaryotic target proteins with high specificity. In most
cases, they grossly affect the biological functions of their
targets. These properties are the reason for using the toxins
as pharmacological tools (Aktories 2000). This applies
especially to C3-like exoenzymes, whose pathogenetic role
is still not well understood. Because C3 toxins modify
selectively RhoA, B, and C, and thereby inactivate the
molecular switches, they have been applied as pharmaco-
logical tools to elucidate the cellular functions of Rho
GTPases in hundreds of papers for more than 20 years. To
obtain more insights into the mode of action of these highly
efficient toxins enhanced structural and biochemical ana-
lyses of C3 exoenzymes have been performed over the last
few years. In this short review we will focus on the
structure and function of C3-like ADP-ribosyltransferases.
Moreover, the recently identified non-enzymatic interaction
of C3 toxins with Ral GTPases is discussed and application
of C3-like ADP-ribosyltransferases as pharmacological and
cell biological tools is briefly reviewed.

Origin of C3-like ADP-ribosyltransferases

Clostridium botulinum C3 transferase ("C3bot1"), the
prototype of C3-like exoenzymes, was initially discovered
in strains of type C and D nearly 20 years ago (Aktories et

al. 1987, 1988; Rubin et al. 1988). The term C3 indicates the third protein toxin beside the C1 neurotoxin and the binary actin-modifying C2 toxin, which are produced by *Clostridium botulinum*. C3bot2, an isoform of the C3 transferase with an identity of ∼65% was identified at nearly the same time (Nemoto et al. 1991). Together with botulinum neurotoxin C1 or D, C3 transferase (e.g., C3bot1) from *Clostridium botulinum* (e.g., strain C-468) is phage-encoded (Popoff et al. 1990; Sakaguchi et al. 2005). A second clostridial C3-like exoenzyme was found in *Clostridium limosum* some years later (Just et al. 1992).

However, expression of C3 transferases is not restricted to clostridia. Four of the seven toxins identified so far are produced by Gram-positive pathogens other than clostridia: *Bacillus cereus* produces the C3cer transferase with a sequence identity of ∼35% to C3bot1 (Just et al. 1995a). Three C3-like enzymes (originally designated EDIN) have been described from *Staphylococcus aureus* and termed in analogy with the other C3-like exoenzymes C3stau1, 2, and 3 (Inoue et al. 1991; Wilde et al. 2001; Yamaguchi et al. 2001). These exoenzymes are ∼35% identical with C3bot1 and 66–77% identical among each other (Fig. 1).

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            1            10           20           30           40           50           60
C3bot1  .........AYSNTYQEFTNIDQAKAWGNAQYKKYGLSKSEKEAIVSYTKSASEINGKLRQNKGVINGF
C3bot2  .........SYADTFTEFTNVEEAKKWGNAQYKKYGLSKPEQEAIKFYTRDASKINGPLRANQGNENGL
C3lim   .........PYADSFKEFTNIDEEARAWGDKQFAKYKLSSSEKNALTIYTRNAARINGPLRANQGNTNGL
C3cer   GNIPTKPKDCNNVDKYKLCTNKEEADAWGKKQFNKW...SKEEKSAIRDYTKNARPYNEFLRMHAGKLDSD
C3stau1 ...........ADVKNFTDLDEATKWGNKLIKQAKYSSDDKIALYEYTKDSSKINGPLRLAGGDINKL
C3stau2 ...........AETKNFTDLVEATKWGNSLIKSAKYSSKDKMAIYNYTKNSSPINTPLRSANGDVNKL
C3stau3 ..........ADDVKNFTDLTEATNWGNKLIKQANYSSKDKEAIYNYTKYSSPINTPLRSSQGDISNF

            70           80           90          100          110          120
C3bot1  PSNLIKQVELLDKSFNK..MKTPENIMLFRGDDPAYLG..TEFQNTLLN.....SNGTINKTAFEKAKAK
C3bot2  PADILQKVKLIDQSFSK..MKMPQNIILFRGDDPAYLG..PEFQDKILN.....KDGTINKTVFEQVKAK
C3lim   PADIRKEVEQIDKSFTK..MQTPENIILFRGDDPGYLG..PDFENTILN.....RDGTINKAVFEQVKLR
C3cer   PT.MKKIESLDKALNRKEAKVNDNIKVYRGDDAWIFG..KEYDNSIIK.....NGKVDREKFKEIQKK
C3stau1 DSTTQDKVRRLDSSISK..STTPESVYVYRLLNLDYLTSIVGFTNEDLYKLQQTNNGQYDENLVRKLNNV
C3stau2 SENIQEQVRQLDSTISK..SVTPDSVYVYRLLNLDYLSSITGFTREDLHMLQQTNNGQYNEALVSKLNNL
C3stau3 SADLQEKILRLDRLISK..SSTSDSVYVYRLLNLDYLSSVKGFSSEDLELLYKTENGKYNEELVKKLNNI
                                            *
                                            R

                   STS   PN                                  ARTT
            130          140          150          160          170          180
C3bot1  FLNKDRLEYGYISTSLMNVSQFAG.RPIITKFKVAKGSKAGYIDP..ISAFAGQLEMLLPRHSTYHIDDM
C3bot2  FLKKDRTEYGYISTSLMS.AQFGG.RPIVTKFKVTNGSKGGYIDP..ISYFPGQLEVLLPRNNSYYISDM
C3lim   FKGKDRKEYGYISTSLVNGSAFAG.RPIITKFKVLDGSKAGYIEP..ISTFKGQLEVLLPRSSTYTISDM
C3cer   FQGKTTTEFGYISTSILIDAGYAKTRPVMTEFKVGSGTHGAYMNSDDLTAYPGQYELLLPRNTVYKIEKI
C3stau1 MNSRIYREDGYSSTQLVSGAAVGG.RPIELRLELPKGTKAAYLNSKDLTAYPGQQEVLLPRGTEYAVGSV
C3stau2 MNSRIYRENGYSSTQLVSGAALAG.RPIELKLELPKGTKAAYIDSKELTAYPGQQEVLLPRGTEYAVGSV
C3stau3 MNSKIYTEYGYSSTQLVKGAALAG.RPIELKLQLPKGTKAAYIDSKNLTAYPGQQEILLPRGTDYTINTV
                      *                                            *
                      S                                            E

            190          200          210
C3bot1  RLSSDG....KQIIITATMMGTAINPK
C3bot2  QISPNN....RQIMITAMIFK......
C3lim   QIAPNN....KQIIITALLKR......
C3cer   YIAIDNNTQKEQIKVEATIK.......
C3stau1 ELSNDK....KKIIITAIVFKK.....
C3stau2 KLSDNK....RKIIITAVVFKK.....
C3stau3 KLSDDH....KRILIEGIVFKK.....
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Fig. 1 Alignment of C3-like transferases. The sequences of the seven C3-like ADP-ribosyltransferases are presented. Note: numbering of the C3-like transferases is without signal sequences. *Clostridium botulinum* C3 transferase type I (C3bot1; Acc. No. P15879), *Clostridium botulinum* C3 transferase type II (C3bot2; Acc. No. Q00901), *Clostridium limosum* C3 transferase (C3lim; Acc. No. Q46134), *Bacillus cereus* C3 transferase (C3cer; Acc. No. AJ429241.1), *Staphylococcus aureus* C3 transferase A, B, and C (C3stau1; Acc. No. P24121; C3stau2; Acc. No. BAC22946, C3stau3; Acc. No.;. NP-478345; also termed EDIN A, B, and C). Important motifs are highlighted: the ADP-ribosylation toxin-turn-turn (*ARTT*) loop is involved in protein substrate recognition and harbors conserved residues essential for the enzymatic activity (C3bot[Q172] and C3bot[E174]); the phosphate-nicotinamide loop (PN-loop), which is involved in the binding of NAD; the STS-motif, which is conserved within the family of ADP-ribosyltransferases (C3stau isoforms possess an STQ motif), is considered to construct a β-strand-α-helix structure to maintain the reaction cavity; finally, several arginine residues involved in interaction with NAD are shown (C3bot[R51], C3bot[R88] and C3bot[R146]). Comparing the sequences of prokaryotic and eukaryotic ADP-ribosyltransferases three residues are highly conserved among all these transferases: an NAD-interacting arginine residue (*R*), the first serine residues from the STS motif (*S*), and the catalytic glutamate (*E*) forming the RSE-motif of ADP-ribosyltrans-

Enzymatic activity and substrate specificity

C3-like exoenzymes catalyze the transfer of the ADP-ribose moiety from the co-substrate NAD to their substrate proteins RhoA, B, and C of the Rho family of low molecular mass GTPases (Fig. 2). Rho^{N41} was identified as acceptor amino acid for the ADP-ribosylation of RhoA, B, and C (Sekine et al. 1989). Other Rho-family members are poor (Rac for C3bot) or marginal substrates (Cdc42 for C3lim) for C3-like ADP-ribosyltransferases (Just et al. 1992; Wilde et al. 2003). The highest specificity for RhoA, B, and C was observed with C3cer (Wilde et al. 2003). The asparagine modification is a unique property of C3 toxins, since many bacterial ADP-ribosyltransferases modify arginine residues (e.g., cholera toxin, *Pseudomonas exoenzyme* S and T, *C. botulinum* C2 toxin; Moss and Vaughan 1977; Barbieri and Sun 2004; Vandekerckhove et

al. 1988) or a cysteine residue (e.g., pertussis toxin; West et al. 1985). Recently, it was shown that the three isoforms C3stau1, 2, and 3 of *Staphylococcus aureus* differ in their substrate specificity. In addition to RhoA, B, and C they also modify the atypical Rho family member RhoE/Rnd3 (Wilde et al. 2001). RhoE and Rnd3 are constitutively active isoforms, which differ only in an addition of 15 amino acids in the N-terminus of Rnd3 and function as a RhoA antagonist (Foster et al. 1996; Wennerberg et al. 2003; Riento et al. 2003). However, the modification of RhoE/Rnd3 has a very slow reaction velocity compared with the ADP-ribosylation of RhoA and the functional relevance is not clear so far.

In the absence of the protein substrates, Rho C3-like ADP-ribosyltransferases hydrolyze NAD into ADP-ribose and nicotinamide (Wilde et al. 2002b). NAD-glycohydrolase activity is also observed with other bacterial ADP-
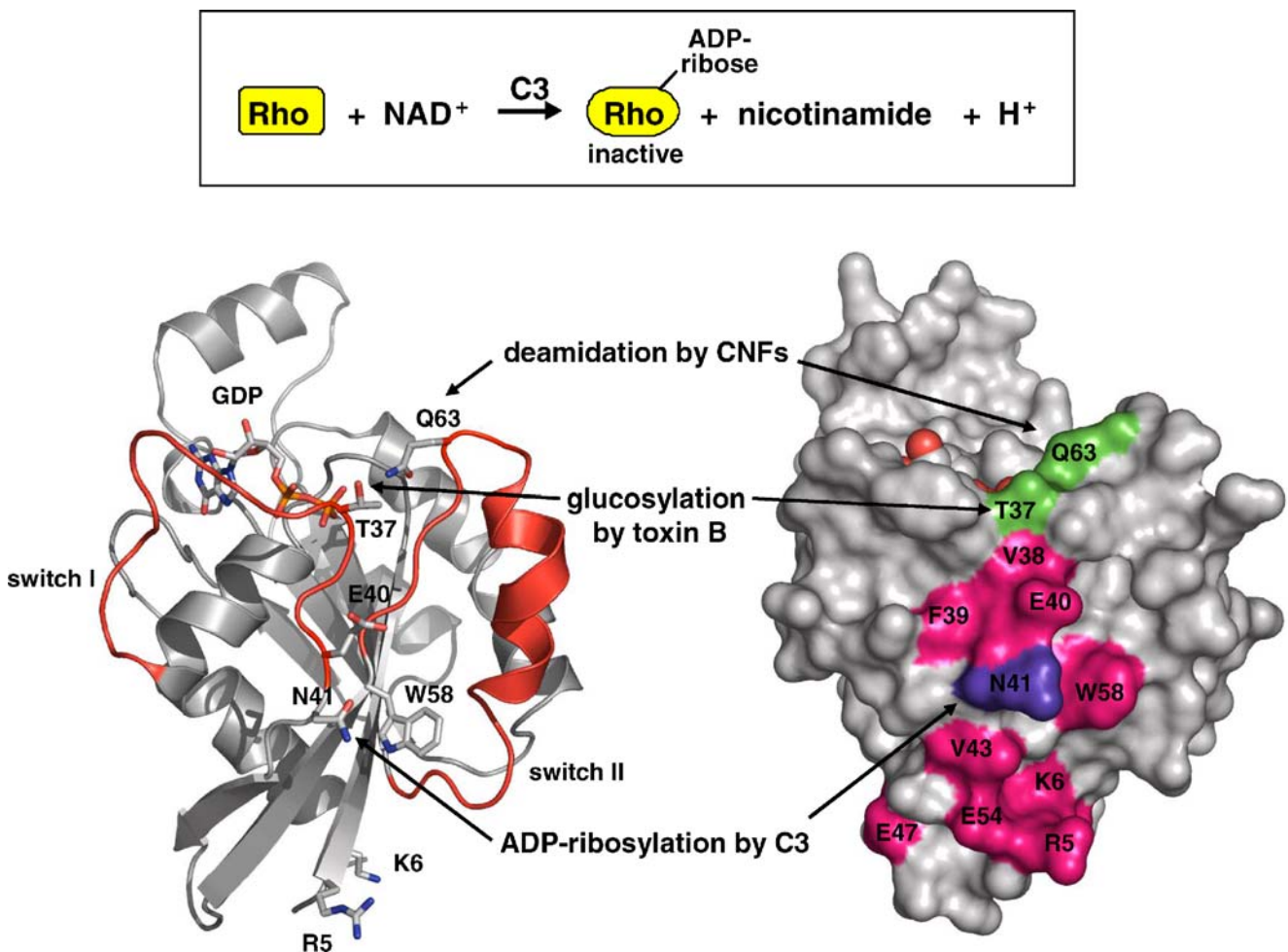
Fig. 2 ADP-ribosylation of RhoA by C3. C3 transferases ADP-ribosylate RhoA at RhoA^{N41} (scheme). RhoA (pdb code 1FTN) is shown as a ribbon plot (*left*) and as a surface representation (*right*) with GDP as a space-filling model. The acceptor RhoA^{N41} (*blue*) is located adjacent to the switch I region in the "effector loop", a region involved in the interaction with effector proteins of RhoA. RhoA^{R5,} $^{K6, E40, E47, E54, W58}$, and RhoA$^{V38, F39, V43}$, which are involved in substrate–toxin interaction, are shown in *pink*. Further residues of RhoA, which are modified by bacterial toxins, are shown in *green*: RhoA^{T37}, which is located in the switch I region, is mono-O-glucosylated by clostridial glucosylating toxins (e.g., *C. difficile* toxin A and B). This leads to inactivation of Rho GTPases. Deamidation of RhoA^{Q63}, which is located in the switch II region, by *E. coli* cytotoxic necrotizing factors (*CNFs*) leads to constitutive activation of RhoA

ribosylating toxins. However, its pathophysiological role is not clear.

The targets of C3-like exoenzymes are molecular switches

Rho proteins represent a discrete branch of the Ras superfamily of low molecular mass GTP-binding proteins and act as molecular switches in various signaling pathways (Jaffe and Hall 2005; Etienne-Manneville and Hall 2002; Burridge and Wennerberg 2004). The Rho subfamily consists of more than 20 members, which share 50% sequence identity and are ubiquitously expressed (Ridley 2000; Wennerberg and Der 2004). After binding of GTP Rho proteins are in the active conformation and inactive when bound to GDP (Fig. 3). Activation occurs by nucleotide exchange of Rho-GDP, which is catalyzed by guanine nucleotide exchange factors (GEFs; Rossman et al. 2005) or inhibited by the binding of guanine

nucleotide dissociation inhibitors (GDIs; DerMardirossian and Bokoch 2005). Up to now, more than 60 GEF and only 3 GDI proteins for Rho have been identified. In the active GTP-bound form Rho proteins mediate various signals by interaction with a specific set of effector proteins. Rho proteins are master regulators of the actin cytoskeleton (Burridge and Wennerberg 2004; Hall 1998). Moreover, they are involved in 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). The signal transduction of Rho is terminated by intrinsic hydrolysis of Rho-GTP, which is catalyzed by GTPase activating proteins (GAPs). More than 70 GAPs have been identified up to now. In the inactive GDP-bound form Rho proteins are extracted from the membrane by GDI, resulting in cytosolic accumulation of an inactive Rho–RhoGDI complex (Moon and Zheng 2003).
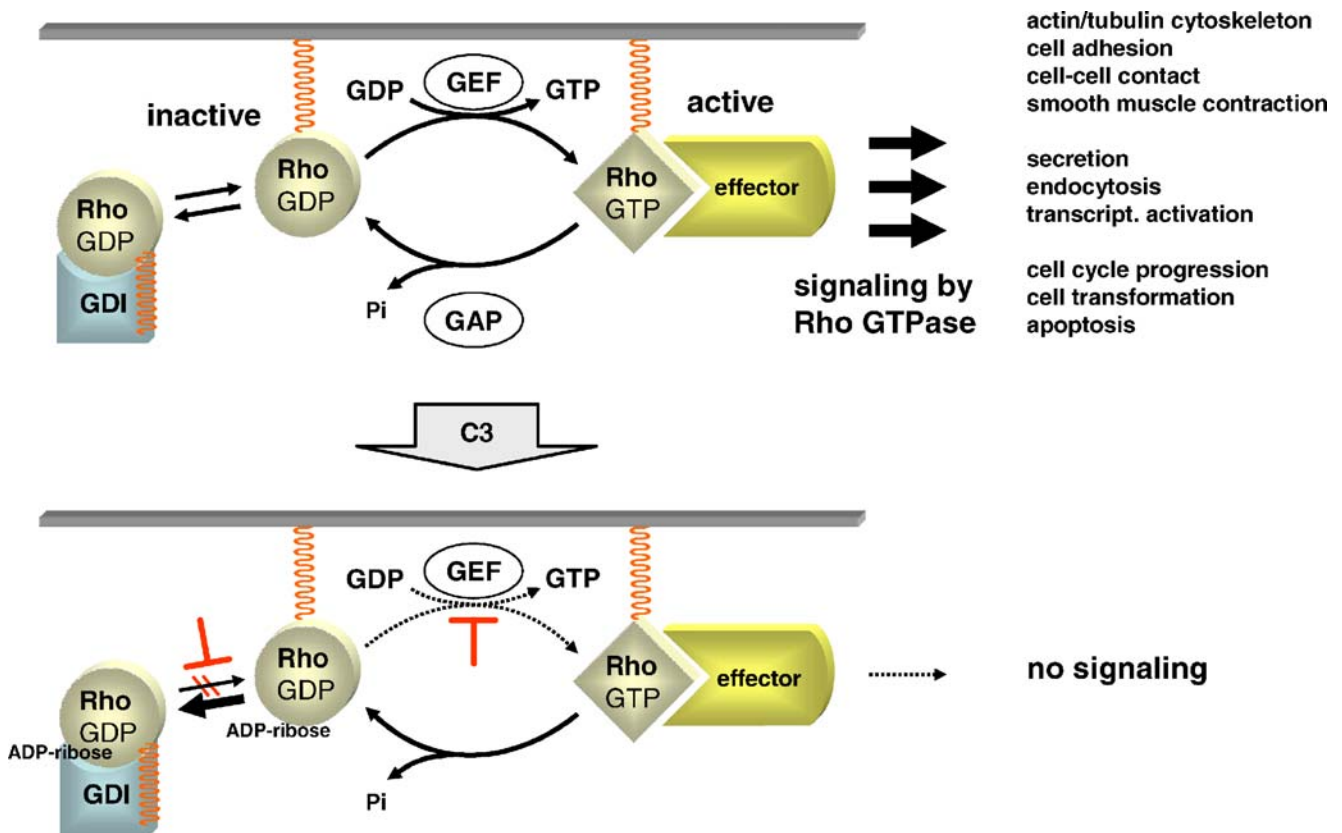
Fig. 3 RhoA GTPase cycle and functional consequences of ADP-ribosylation by C3. Rho GTPases are inactive in the GDP-bound form and activated by GDP/GTP exchange, which is facilitated by guanine nucleotide exchange factors (GEFs). In the active form RhoA GTP interacts with a large variety of effectors to induce various cellular effects indicated. The active form of RhoA is terminated by hydrolysis of the bound GTP, which is enhanced by GTPase-activating proteins (GAPs). The inactive form is extracted from the membrane by guanine nucleotide exchange factors (GDI), which stabilize Rho GTPases in an inactive Rho–RhoGDI complex in the cytosol. RhoA is ADP-ribosylated by C3 in the GDI-free form and is then inactivated by a two-step mechanism: 1. ADP-ribosylation inhibits the activation of RhoA by GEF. 2. Binding of ADP-ribosylated RhoA to GDI is increased, which leads to a cytosolic accumulation of inactive Rho–RhoGDI complex ("trapping effect")

Studies from recent years identified Rho GTPases as the preferred targets of several bacterial toxins. In addition to the ADP-ribosylation by C3, Rho GTPases are covalently modified by glucosylation (e.g., by clostridial glucosylating toxins; Aktories and Barbieri 2005; Just et al. 1995b; Aktories and Just 2005; Voth and Ballard 2005; Just and Gerhard 2004), deamidation (e.g., by *E. coli* cytotoxic necrotizing factor; Schmidt et al. 1997; Flatau et al. 1997), and transglutamination (*Bordetella* dermonecrotizing factor; Masuda et al. 2000) resulting in inactivation or activation of the GTPases respectively (Fig. 2). Other toxins influence the activity of Rho GTPases by mimicry of endogenous GAP activity, e.g., *Yersinia* YopE (von Pawel-Rammingen et al. 2000), *Salmonella* SptP (Fu and Galan 1999), *Pseudomonas aeruginosa* ExoS (Goehring et al. 1999) or endogenous GEF activity, e.g., *Salmonella* SopE (Hardt et al. 1998). Finally, it has been shown recently that Rho GTPase were cleaved proteolytically at the C-terminal isoprenylated cysteine by *Yersinia* YopT and thereby inactivated (Shao et al. 2002, 2003).

Functional consequence of the ADP-ribosylation

C3-like exoenzymes catalyze the transfer of the ADP-ribose moiety of NAD on a conserved asparagine residue in Rho (e.g., RhoA^{N41}), which is located directly adjacent to the so-called switch I region (Fig. 2; Sekine et al. 1989; Maehama et al. 1994). The switch regions are structural hallmarks of guanine nucleotide-binding proteins and undergo dramatic conformational changes upon nucleotide exchange, which is required for the downstream signaling (Vetter and Wittinghofer 2001). ADP-ribosylation of this asparagine residue renders Rho biologically inactive and leads to a dramatic and lethal redistribution of actin in the target cell as a consequence (Chardin et al. 1989; Paterson et al. 1990; Wiegers et al. 1991). The mechanism underlying the inactivation of Rho by C3-mediated ADP-ribosylation was analyzed in great detail. First, it was shown that the ADP-ribosylation does not influence nucleotide-binding and hydrolysis (Sehr et al. 1998). This finding is in line with the crystal structure of RhoA-GTP, where RhoA^{N41} is surface located and does not interact with the nucleotide. Moreover, several crystal structures of Rho in complex with effector or regulator proteins respectively have revealed RhoA^{N41} as part of a conserved interaction site of low molecular GTP binding proteins (Dvorsky and Ahmadian 2004). It was thus reasonable to assume that ADP-ribosylation inactivates RhoA by inhibition of downstream signaling (i.e., blocking Rho-effector interaction) or by affecting the Rho-regulator interaction. From the follow-up studies it turned out that ADP-ribosylation inactivates RhoA by at least two steps: first

by inhibition of the GEF-mediated nucleotide exchange of RhoA (e.g., Lbc; Sehr et al. 1998) and second by the trapping of ADP-ribosylated RhoA in the inactive RhoA–RhoGDI complex in the cytosol (Fig. 3; Genth et al. 2003a; Fujihara et al. 1997). Accordingly, translocation and activation of RhoA under physiological conditions is effectively blocked after ADP-ribosylation by C3-like transferases. However, inactivation of Rho by ADP-ribosylation can be overcome by treatment with the cytotoxic necrotizing factor CNF1. CNF1 deamidates RhoA at RhoA^{Q63}, which is located in the switch II region, and thereby constitutively activates RhoA (Barth et al. 1999). In contrast, no evidence was found for the hypothesis that ADP-ribosylation prevents binding and activation of downstream effectors (Sehr et al. 1998; Genth et al. 2003b) or influences the GAP-mediated Rho inactivation (Sehr et al. 1998).

The pathogenetic role of C3-like exoenzymes

The pathogenetic role of C3-like exoenzymes is not well understood. One reason is the enigmatic up-take mechanism of the exoenzymes. Their structure is not in line with the composition of typical AB toxins, which possess an enzyme domain and a cell binding and translocation domain. C3-like toxins lack a specific transportation unit and, therefore, their cell accessibility is poor in many cell lines.

At least three possibilities for an effective uptake into their target cells are discussed. First, the concentration of C3 in the surrounding of the toxin-producing bacterium is very high allowing entry of the toxin into target cells over an extended time by unspecific up-take. Second, C3 toxins may be taken up by means of an additional bacterial pore-forming factor according to the mechanism proposed for the action of the *Streptococcus pyogenes* NAD glycohydrolase SPN. SPN appears to be specifically translocated into host cells by the pore-forming cytolysin Streptolysin O (Madden et al. 2001; Meehl and Caparon 2004). It remains to be clarified whether pore-forming cytolysins, which are frequently synthesized by C3-producing clostridia, are involved in the translocation of C3. Finally, at least for C3-producing *Staphylococcus aureus* an alternative mechanism has been suggested. For these bacteria, which are classically considered as extracellular pathogens, it was shown that they can act as intracellular pathogens, capable of invading and surviving within a broad range of non-phagocytic cells, including enterocytes, and endothelial and epithelial cells (Hess et al. 2003; Lowy 2000). Accordingly, recent studies indicate that these bacteria are able to invade host cells and secrete the C3 toxins directly into the host cytosol. Thus, it was shown that C3stau2 reaches the

cytosol of the target cell during the staphylococcal invasion process without the need of a cell membrane translocation machinery (Molinari et al. 2006).

Once in the cytosol, C3 toxins should affect numerous Rho-dependent signal pathways in a manner as mentioned above. From a pathophysiological point of view it is important that C3 was shown to alter for example epithelial and endothelial barrier functions (Nusrat et al. 1995; Stamatovic et al. 2003), the signaling of immune cells including phagocytosis (Caron and Hall 1998), the production of cytokines (Chen et al. 2002; Dreikhausen et al. 2001), adhesion (Laudanna et al. 1996), de-adhesion (Liu et al. 2002), and migration of immune cells (Worthylake et al. 2001; Millan and Ridley 2005). A very recent study reported that C3stau-producing *S. aureus* form transcellular tunnels by inactivation of Rho, so-called macroapertures, which lead to the loss of barrier function in endothelial cells (Boyer et al. 2006). Other functions of Rho GTPases in immune cells, which are potentially targeted by bacterial toxins including C3 exoenzymes, have been reviewed recently (Aktories and Barbieri 2005; Cantrell 2003; Bokoch 2005).

C3 toxins are pharmacological tools

Because of the high specificity for Rho GTPases and their effective inactivation by ADP-ribosylation, C3-like exoenzymes were widely used as Rho inhibitors in many pharmacological and cell biological studies (Aktories et al. 2004). The application of C3-like ADP-ribosyltransferases as tools is limited by their poor cell accessibility. To overcome the poor cell accessibility of C3-like ADP-ribosyltransferase several strategies were successfully applied. Quite often the toxins were introduced by microinjection (Paterson et al. 1990; Watanabe et al. 1997). Furthermore, several C3 toxin chimeras were generated. In one of the earlier approaches, C3bot was fused to the binding and translocation subunit of diphtheria toxin resulting in the construct DC3B (Aullo et al. 1993). In another approach the binary actin-ADP-ribosylating C2 toxin from *Clostridium botulinum* was employed to construct a chimeric fusion toxin. C2 toxin consists of the actin-ADP-ribosylating enzyme component C2I and the binding and translocation component C2II, which are both separated proteins (Ohishi et al. 1980; Aktories et al. 1986; Aktories and Wegner 1989). The N-terminal part (C2IN) of C2I, which interacts with the binding component C2II, was fused to full length C3lim or C3stau respectively (Haug et al. 2006; Barth et al. 1998). All mammalian cells studied so far are sensitive towards the C2–C3 fusion toxin (e.g., CHO, Hela and NIH-3T3 cells; Valderrama et al. 2001; Wahl et al. 2000; Meyer et al. 2000; Vischer et al. 2000).

Typical cytotoxic effects were observed at 200 ng/ml C2IN-C3lim/C3stau and 200 ng/ml C2II after 1–2 h of incubation (Barth et al. 1998). Several studies have shown that the C-terminal fusion of short transport peptide sequences enhances the cellular uptake of C3 (Sauzeau et al. 2001; Park et al. 2003; Winton et al. 2002; Sahai and Olson 2006). In many studies, the C3 gene was introduced into eukaryotic target cells by transient and stable transfection using plasmids or by viral infection (Fujisawa et al. 1998; Hill et al. 1995; Caron and Hall 1998; Henning et al. 1997; Meacci et al. 1999; Genot et al. 1996).

Structural analysis of C3-like exoenzymes

Crystal structure analysis using X-ray diffraction is one of the most advanced methods available for obtaining high resolution structural information about biological macromolecules. In case of the C3-like exoenzymes, the structures of two family members have been solved so far.

C3bot1 was the first C3-like exoenzyme, which was successfully crystallized by Han et al. (2001). The overall structure of the toxin has a mixed α, β-fold: a central β-sandwich, which is formed by a perpendicular packing of a five-stranded β-sheet against a three stranded β-sheet surrounding the NAD-binding pocket (Fig. 4). Important for the architecture of this pocket are three residues C3bot$^{S134, T135}$ and C3bot^{S136}, which are conserved in all C3-like transferases (Note: numbering of C3bot is without the signal sequence). Together they form the so-called STS motif (in C3stau transferases an ST motif), which connects strand β3 with the PN loop and maintains the reaction cavity. This central cleft of C3bot is terminated on one side by a so called ARTT loop (Han et al. 2001), a double turn motif consisting of 10 residues. The ARTT loop was proposed as part of the active site center and is conserved in other ADP-ribosyltransferases (e.g., actin-modifying *Bacillus cereus* VIP 2). It contains the invariant catalytic glutamate C3bot^{E174} as well as C3bot^{Q172} and C3bot^{F169} residues, which are necessary for the activity of C3-like transferases. In their model for the ADP-ribosylation of Rho by C3bot, Tainer and coworkers predicted that C3 recognizes its substrate mainly via the ARTT loop (Han et al. 2001). In that context C3bot^{F169} was proposed to contribute to the binding of Rho via a hydrophobic interaction with a patch of Rho (RhoA^{V38,F39,V43}), whereas C3bot^{Q172} was proposed to interact with Rho^{N41}, the acceptor for the ADP-ribose moiety.

Ménétrey and coworkers compared structures of monomeric C3bot and C3bot complexed to NAD (Ménétrey et al. 2002). NAD binds in an elongated conformation into the central cleft of C3bot and contacts the ARTT loop as well as a further structural element, the so-called phosphate PN
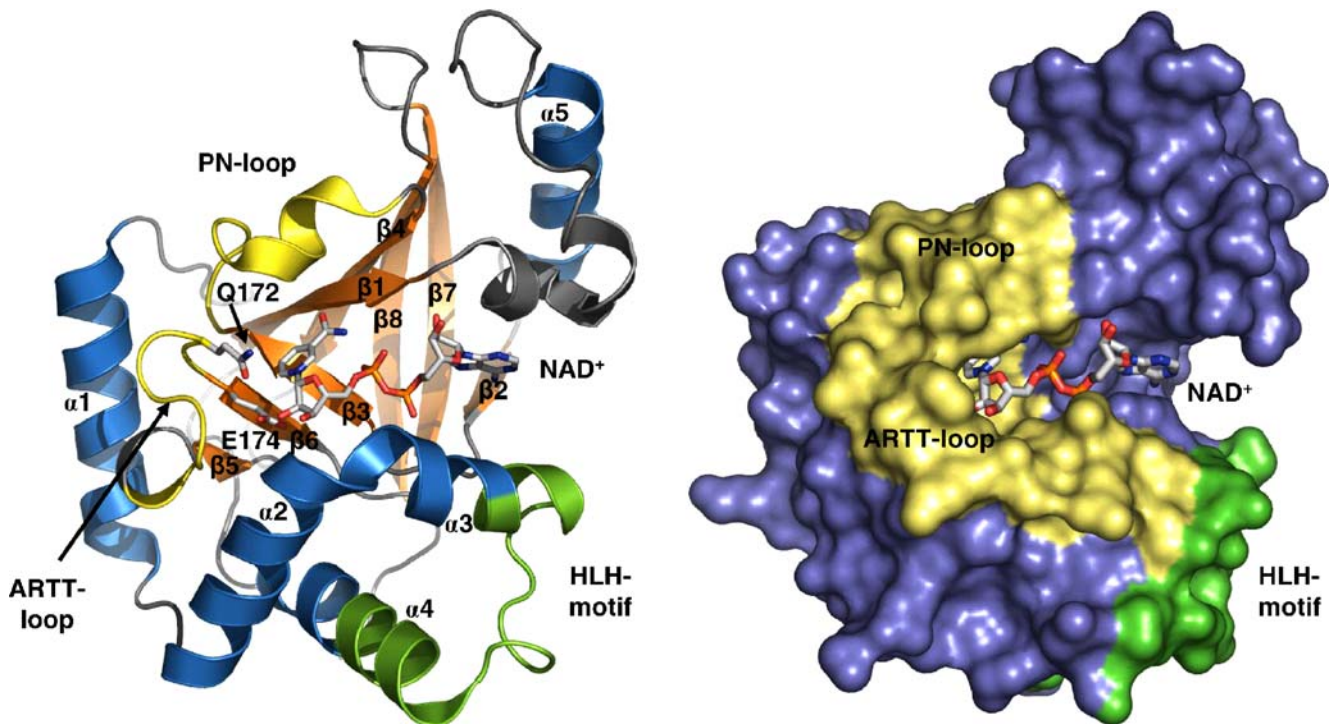
Fig. 4 Structure of *C. botulinum* C3 transferase (C3bot1). C3bot in complex with NAD (pdb code 1GZF) is displayed as ribbon plot (*left*). Secondary structure elements are color-coded (α-helices, *blue*, β-sheets, *orange*) and labeled according to Han et al. (2001). ARTT loop and PN loop, which enclose the active site are shown in *yellow*, with the catalytic residues C3bot^{Q172} and C3bot^{E174} as stick models; the newly defined helix-loop-helix motif (HLH motif, *green*) mediates the non-enzymatic interaction of C3bot with Ral. A surface representation (*right*) indicates the Ral binding site (*green*) and three regions that are important for Rho recognition (*yellow*)

loop (Fig. 4). The PN loop (C3bot$^{137-146}$) stabilizes NAD by interacting with its nicotinamide moiety and by compensating the charge of one phosphate group with a conserved arginine residue. The structural comparison of the monomeric C3bot and the C3bot-NAD complex revealed that binding of the cosubstrate NAD induces conformational changes in the C3 exoenzyme. The ARTT loop underwent an "ample swinging motion" into the NAD-binding pocket. Most prominently, C3bot^{Q172} was found to translocate from a surface exposed to a buried location. In the remodeled NAD-binding pocket C3bot^{Q172} was then found to interact with the O-2′-hydroxyl ribose of the nicotinamide ribose (Fig. 4). The conformational change of C3bot^{Q172} is thought to be important for correctly positioning Rho into the ternary Rho-NAD-C3 complex (Han et al. 2001; Ménétrey et al. 2002). Additionally, the catalytic glutamate C3bot^{E174} was found to interact with O-2′-hydroxyl ribose of the nicotinamide ribose, which suggested the stabilization of an oxocarbenium transition state during the ADP-ribose transfer.

An analysis of overall conformational changes was reported by Evans et al. (2003). Comparing all available structural data, they concluded that the molecule possesses a rigid core formed largely from the β-strands and significant main-chain deviations are restricted to peripheral substructures, namely the ARTT loop, the PN loop, and the C-terminal end of the β1–α5 loop.

Recently, crystal structures of *S. aureus* C3stau2 were solved in the absence and presence of NAD (Evans et al. 2003). C3stau2 has a broadened substrate specificity compared with the other C3-like ADP-ribosyltransferases; in addition to RhoA, B, and C it also ADP-ribosylates the atypical Rho family member RhoE (Wilde et al. 2001).

The overall topology of C3stau2 is similar to that of C3bot despite a sequence similarity of only 35%. A deviation is found after strand β1, where C3stau2 has inserted two additional α-helices. A major difference between C3stau2 and C3bot1 was found in the conformation of the ARTT loop: in contrast to the cosubstrate-induced conformational changes observed for C3bot, the ARTT loop of C3stau, which contains two additional residues, folds in a fixed conformation in the absence and presence of NAD similar to that of the C3bot-NAD complex. Significant conformational changes after binding of NAD in C3stau were only found for the PN loop. A further difference in the structure of C3stau was the position of helix α3 from the helical bundle in comparison to C3bot. Together with the difference in length and

conformation of the ARTT loop this could contribute to the additional modification of RhoE by C3stau2.

Structure–function analysis of the ADP-ribosylation of Rho by C3-like exoenzymes

Beside the crystallographic analysis a detailed structure–function analysis of C3-like exoenzymes (and RhoA) has been performed over the last few years. The available structural data in combination with a detailed biochemical analysis provides precise information about the binding of NAD, the substrate recognition and the mode of action of ADP-ribosylation of C3-like ADP-ribosyltransferases. Some important residues and their function in C3 toxins as well as in the substrate RhoA are discussed in the following paragraph.

The structural analysis showed that a conserved glutamate residue, the so-called catalytic glutamate, is involved in the binding of the nicotinamide ribose of NAD by hydrogen bonds with the O2-hydroxyl group (Fig. 4). Furthermore, this glutamate residue was proposed to stabilize an oxocarbenium transition state during the ADP-ribose transfer. The biochemical analysis supported the essential role of this residue for the enzymatic activity. Exchange of the catalytic glutamate in C3-like ADP-ribosyltransferases blocked the ADP-ribosylation of RhoA as well as the NAD hydrolysis in the absence of RhoA (Aktories et al. 1995; Böhmer et al. 1996; Wilde et al. 2002b, 2003; Han et al. 2001).

A conserved glutamine residue is located in turn 2 of the ARTT loop in all C3-like ADP-ribosyltransferases separated by a variable amino acid "X" from the catalytic glutamate. This sequence was termed QXE motif and is typical of non-arginine-modifying ADP-ribosyltransferases, e.g., the asparagine-modifying C3-like ADP-ribosyltransferases (Fig. 1). Exchange of this glutamine residue to alanine or glutamate blocked ADP-ribosylation of Rho; surprisingly, the binding of Rho and NAD and hydrolysis of NAD were hardly affected in these mutant proteins (Ménétrey et al. 2002; Wilde et al. 2002b, 2003). Building on the structural data a role of the glutamine residue in positioning of the acceptor RhoA[N41] during formation of the Rho–C3 complex was proposed (Wilde et al. 2002b, 2003). The function of the glutamine residue was analyzed in more detail recently. Exchange to glutamate, which is at the respective position in several arginine-modifying ADP-ribosyltransferases blocked the asparagine modification of RhoA (Vogelsgesang and Aktories 2006). However, this glutamine–glutamate mutation in several C3-like transferases (e.g., C3bot[Q172E] or EXE-C3bot) allowed ADP-ribosylation of arginine residues by the enzymes. Thus, the single mutation of the QXE motif into an EXE motif changes the specificity of C3-like ADP-ribosyltransferases from asparagine to arginine. The results indicate that the glutamine residue of the QXE motif stabilizes the acceptor asparagine in RhoA (RhoA[N41]) during the ADP-ribosylation reaction (Fig. 5).

The structural prerequisites of RhoA for the recognition by C3bot were analyzed by (Wilde et al. 2000). The region
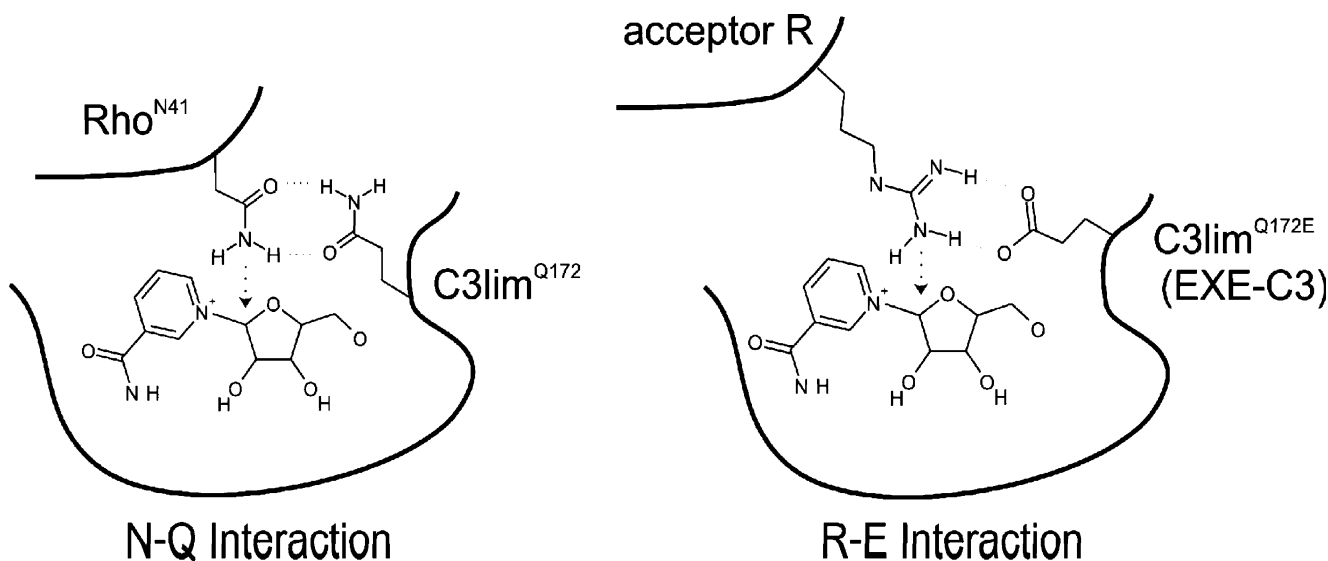
Fig. 5 Model for the interaction of asparagine or arginine with WT-C3 (QXE)- or EXE-C3 (e.g., C3lim[Q172E]). A scheme of the catalytic core of C3, containing the nicotinamide ribose part of NAD is shown. C3lim[Q172], which is located in the ARTT loop, recognizes RhoA[N41] via two hydrogen bonds. Due to the Q–N interaction RhoA[N41] is in a favorable position for a nucleophilic attack on C1 of the ribose (asterisks). EXE-C3lim recognizes arginine residues. This R–E interaction is favorable because it takes advantage of the polar and electrostatic characteristics of both amino acids and allows ADP-ribosylation. H-bonds are not necessary, but support the recognition of arginine residues by EXE-C3lim

for the interaction of RhoA with C3bot was restricted to the N-terminal half of RhoA by generating chimeric proteins between RhoA and the non-substrate Rac1. Within the N-terminal half, an alignment of Rho and the non-substrate Rac revealed remarkable amino acid differences in the two regions: RhoA[R5, K6] and RhoA[E40, E47, E54] respectively. Subsequent introduction of RhoA[R5, K6, E40, E47, E54] at the equivalent positions in Rac1 allows its ADP-ribosylation by C3bot with kinetics comparable to the modification of RhoA. Interestingly, it has been speculated that RhoA[R5, E40] may have a general impact for the enzyme–protein substrate interaction of ADP-ribosyltransferases, since functional homolog residues were identified at the interface in the crystal structure of eukaryotic elongation factor 2 (eEF2) in complex with the ADP-ribosylating toxin exotoxin A from *Pseudomonas aeruginosa* (Jorgensen et al. 2005). Beside their putative role in substrate recognition, RhoA[E40] stabilizes the acceptor amino acid RhoA[N41] via hydrogen bonds on the surface of RhoA-GTP (Ihara et al. 1998). This interaction brings the side chain of RhoA[N41] into a close vicinity with the indole ring of Rho[W58], which may enhance the nucleophilicity of RhoA[N41] for the ADP-ribosylation.

Further important residues of Rho for the interaction with C3 are RhoA[V38, F39] and RhoA[V43]. Their side chains form a hydrophobic ring around RhoA[N41], which is suggested to interact with the conserved F/Y residue of C3 (Han et al. 2001). Thus, exchange of the respective residues inhibited ADP-ribosylation of the mutant proteins (Wilde et al. 2000).

Non-enzymatic interaction of C3-like exoenzymes with Ral

All C3-like exoenzymes ADP-ribosylate and thereby inactivate specifically RhoA, B, and C from more than 150 identified low molecular GTP-binding proteins. In addition, C3-like ADP-ribosyltransferases interact non-enzymatically with Ral without ADP-ribosylating the GTPase (Wilde et al. 2002a; Pautsch et al. 2005). Ral belongs to the Ras branch of low molecular GTPases and is involved in transcriptional activation (Hernandez-Munoz et al. 2000), Ras-mediated cell transformation (Feig et al. 1996; Urano et al. 1996), vesicle trafficking (Shen et al. 2001) and cytoskeletal rearrangements (Jullien-Flores et al. 1995; Ohta et al. 1999).

The interaction of Ral and C3bot was first identified in initial substrate scans of C3bot. In the presence of Ral, which is not a substrate for C3-like exoenzymes, the ADP-ribosylation of RhoA by C3bot was reduced.

More recently, it was shown that this inhibitory effect was specific for Ral and that Ral binds with high affinity to C3bot and C3lim. In contrast, Ras, which is the closest homolog to Ral, does not interact with C3 (Pautsch et al. 2005). However, ADP-ribosylation of RhoA by C3cer and C3stau2 was less or not influenced in the presence of Ral. Furthermore, C3bot decreased activation of the effector protein phospholipase D by RalA (Wilde et al. 2002a).

The molecular basis of the C3bot–RalA interaction was recently identified by two groups (Holbourn et al. 2005; Pautsch et al. 2005). The structure of a stochiometric RalA (GDP)-C3bot complex with an apparent K_D value of ~60 nM was determined by X-ray crystallography (Pautsch et al. 2005). In the structure of this complex C3bot inserts a previously unrecognized helix-loop-helix motif into a deep pocket formed predominantly by the switch II region and helix α3 of RalA (Fig. 6). The biological relevance of the interface deduced by Pautsch et al. was then verified by mutational analysis (Pautsch et al. 2005). The sequence of the C3 helix-loop-helix motif is less conserved in C3cer and the C3stau isoforms, explaining the specificity of Ral for the two other toxins. Notably, the Ral–C3 interface is located adjacent to regions that are proposed to be involved in Rho recognition (Fig. 4). On the other hand the C3 binding site in Ral does also not correspond with the ADP-ribosylation site in the switch I region of RhoA. In summary, the binding mode of Ral-C3 does not resemble the proposed substrate recognition mode of Rho by C3bot. It is highly likely that Ral inhibits the ADP-ribosylation by high affinity binding to an epitope of C3 in close proximity to the Rho-interaction site. Another possible explanation for the inhibitory effect is that Ral prevents conformational changes in C3bot, which occur during the ADP-ribosylation. It is noteworthy that the crystal structure of the RAL-C3 complex reported by Acharya and coworkers (Holbourn et al. 2005) predicts an interaction of the GTPase with the ARTT loop of C3. However, this model could not be supported by biochemical and mutational data (Pautsch et al. 2005).

The Ral–C3 complex described in Pautsch et al. (2005) suggested a functional consequence for Ral: binding of the helix-loop-helix motif stabilizes the conformation of the switch regions of Ral with respect to the native structures where the switch regions are found disordered (Nicely et al. 2004). Although C3bot does not directly interact with the guanine diphosphate, it stabilizes nucleotide binding indirectly through its interaction with the switch II region of Ral. A similar mode of nucleotide stabilization had been observed previously for two GDIs of the Rab family proteins (Rak et al. 2003, 2004) and for RhoGDI (Hoffman et al. 2000), suggesting that C3bot could act to Ral in a GDI-like manner. A structural comparison of known GDI complexes with Ral amd C3bot is shown in Fig. 6. All three regulators bind to the GTPase effector binding region, but differ in their detailed molecular mode of action.

Fig. 6 GTPase recognition of GDIs and a GDI-like acting toxin. The crystal structures of Ral in complex with C3bot (Pautsch et al. 2005), the Rab-like GTPase Ypt1 in complex with RabGDI (Rak et al. 2003), and Cdc42 with RhoGDI (Hoffman et al. 2000) are shown in an identical orientation relative to the GDP-bound GTPase. All three regulators bind to the GTPase effector binding region, in particular switch II, but differ in their detailed molecular mode of action. GDIs (RhoGDI and RabGDI) and GDI-like toxin C3bot are shown as *blue* ribbons; GTPases are shown in *gray*, with the switch I and switch II regions in *red*. GDP is shown as a stick model. NAD bound to C3bot and the C-terminal geranylgeranyl moiety of Cdc42 are shown as stick models. Residues of the GTPases that interact with the GDI are colored *green*. The molecular mode of the GDI-like stabilization of GDP by C3bot is shown in the *insert*. C3bot is displayed as a cyan worm. RalA is shown in *gray* except for the switch I (*blue*) and switch II (*red*) regions. Interacting residues of RalA-GDP and C3bot are shown in stick representation with atoms other than carbon colored by atom type ©, *yellow*; O, *red*; N, *blue*; P, *magenta*). Water molecules are shown as *red spheres*. The Mg^{2+} ion is presented as a *black sphere*, hydrogen bonds are shown as *yellow dotted lines*
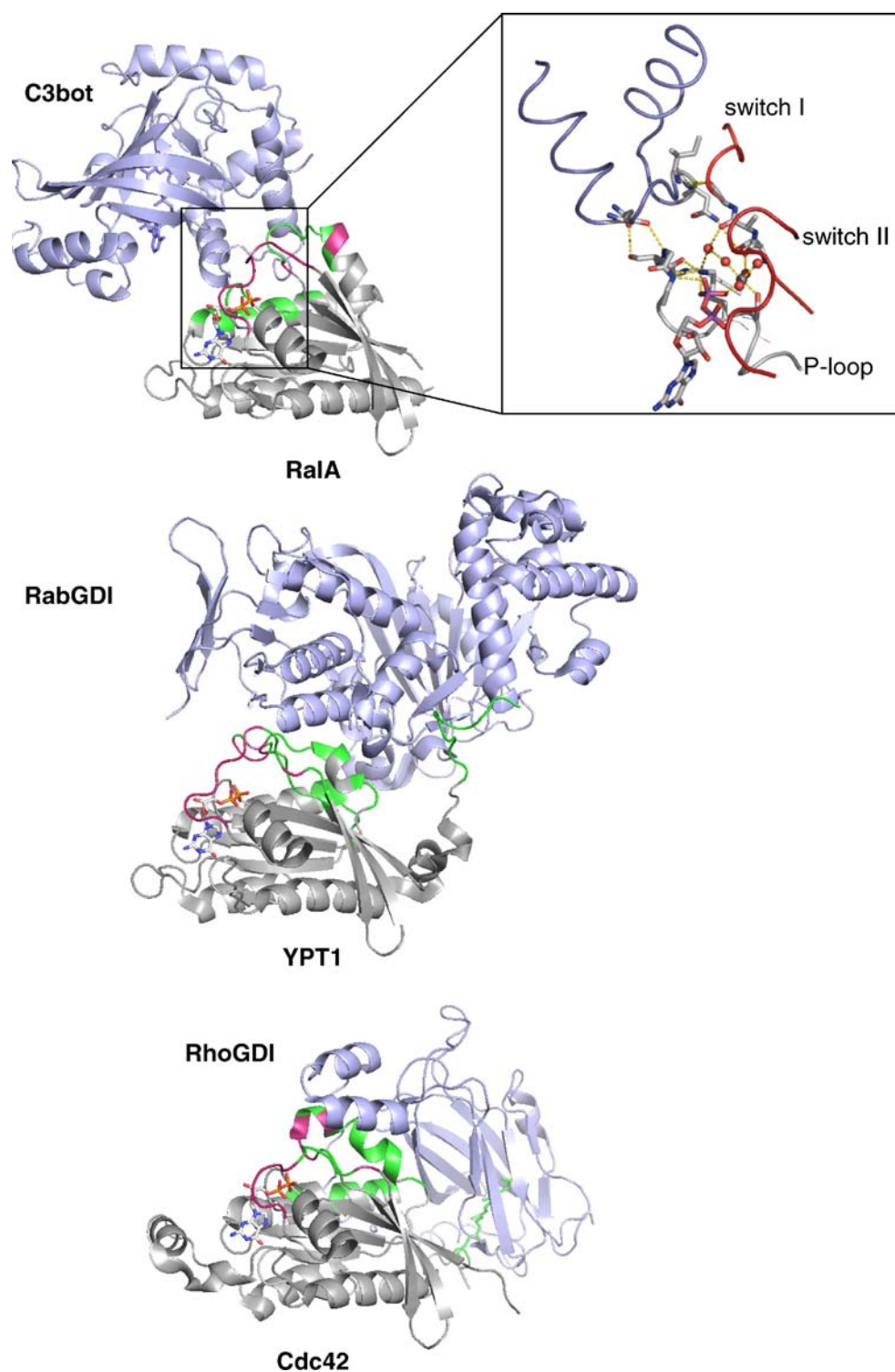
This hypothesis that C3bot exerts a GDI-like effect was corroborated by the biochemical analysis of the nucleotide exchange of Ral with fluorescently labeled mant nucleotides in the presence of C3bot. It turned out that the nucleotide binding of Ral was altered in the Ral–C3bot complex. The addition of C3bot resulted in the concentration-dependent inhibition of the nucleotide release of Ral, which was preloaded with mant GDP. This inhibition of GDP exchange and the subsequent trapping of GDP was previously found with RhoGDI and RhoGTPases and termed GDI effect (Herrmann et al. 1995; Ahmadian et al. 2002). In contrast, C3bot had no influence on the exchange

of mant GTP from Ral. The latter finding was in line with the low affinity for the interaction of Ral(GTP) and C3bot (K_D value >10 μM). Also the structural alignment of the Ral(GDP)–C3bot complex with Ral(GTP) in complex with the effector protein sec5 revealed that the conformation of the switch regions in Ral(GTP) precluded binding to C3bot. Taken together, these data suggest that C3bot is a bifunctional toxin targeting Rho by ADP-ribosylation and Ral by a GDI-like manner, which blocks nucleotide exchange and effector interaction. However, it remains to be shown that the effects of C3 in intact cells are not only caused by inactivation of Rho GTPases, but also by its interaction with Ral.

Other non enzymatic interaction of C3-like exoenzymes

Recently, another C3 effect, which is independent of the ADP-ribosyltransferase activity, has been reported (Ahnert-Hilger et al. 2004). It is well-known that Rho proteins regulate neurite outgrowth (Dergham et al. 2002). Several studies showed that C3 prevents neurite retraction induced by activated RhoA (Grunwald and Klein 2002; Wahl et al. 2000; Lehmann et al. 1999). 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.

Conclusions

The structural and biochemical analysis of C3-like ADP-ribosyltransferases over the few last years has provided significant progress in further understanding the enzymatic and non-enzymatic actions of these toxins. Despite their rather low sequence identity the overall folding of C3 toxins is very similar. The ARTT loop, together with the PN loop and helix α3, are important and conserved structural elements in the active site. From the analysis of the substrate recognition of the related ADP-ribosyltransferases ExoS and ExoT from *Pseudomonas aeruginosa* it was proposed that the recognition of Rho by C3 toxins is mediated by these conserved regions. However, to prove this model it is of major importance to solve the structure of C3 in complex with Rho. Novel properties of C3 toxins were identified deduced from the crystal structure of C3bot in complex with RalA and strengthened by biochemical data. The finding of the GDI-like non-enzymatical interac-

tion of C3bot with Ral offered novel perspectives for the understanding of the mode of action and functional role of C3. Also important for the pathophysiological role of C3 was the finding that C3stau2 was secreted into the cytosol of eukaryotic cells after invasion of the bacteria. These data provide a potential explanation of how the C3-like exoenzymes, which lack a specific transportation unit, were able to enter their target cells.

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