Recognition of RhoA by *Clostridium botulinum* C3 Exoenzyme

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The C3-like ADP-ribosyltransferases exhibit a very confined substrate specificity compared with other Rho-modifying bacterial toxins; they selectively modify the RhoA, -B, and -C isoforms but not other members of the Rho or Ras subfamilies. In this study, the amino acid residues involved in the RhoA substrate recognition by C3 from *Clostridium botulinum* are identified by applying mutational analyses of the nonsubstrate Rac. First, the minimum domain responsible for the recognition by C3 was identified as the N-terminal 90 residues. Second, the combination of the N-terminal basic amino acids (RhoArg^2/-Lys^6), the acid residues RhoGlu^47 and RhoGlu^54 only slightly increases ADP-ribosylation but fully restores the binding of the respective mutant Rac to C3. Third, the residues RhoGlu^50 and RhoVal^43 also participate in binding to C3 but they are mainly involved in the correct formation of the ternary complex between Rho, C3, and NAD^+. Thus, these six residues (Arg^2, Lys^6, Glu^47, Val^43, Glu^50, and Glu^54) distributed over the N-terminal part of Rho are involved in the correct binding of Rho to C3. Mutant Rac harboring these residues shows a kinetic property with regard to ADP-ribosylation, which is identical with that of RhoA. Differences in the conformation of Rho given by the nucleotide occupancy have only minor effects on ADP-ribosylation.

*Clostridium botulinum* exoenzyme C3 is the prototype of the family of C3-like ADP-ribosyltransferases that modify the Rho subtype GTPases. C3 catalyzes the transfer of the ADP-ribose moiety from the co-substrate NAD^+ to asparagine 41 of the RhoA, -B, and -C proteins (1). In addition to various isoforms of Rho (2–4), this family encompasses the exoenzymes from *Clostridium limosum* (5), *Bacillus cereus* (6), and *Staphylococcus aureus*, designated EDIN (epidermal differentiation inhibitor) (7). C3 selectively ADP-ribosylates the Rho isoforms A, B, and C but not other members of the Rho or Ras superfamily (5, 8, 9). Only under a special condition such as the presence of low concentration of sodium dodecyl sulfate is Rac modified to a minor extent (5). In addition to the transferase activity, the C3-like exoenzymes exhibit glycohydrolase activity to cleave NAD^+ in the absence of the protein substrate (10).

The C3-like exoenzymes are single-chain proteins with a molecular mass of about 25 kDa. They lack a membrane binding and translocation domain, and their cell entry is most likely mediated through pinocytosis. This deficiency in cell entry has been overcome by the construction of chimeric C3 toxins, which exploit the cell entry domains of other toxins such as diphtheria or *C. botulinum* C2 toxin (11, 12).

The RhoA, -B, and -C proteins belong to the Rho subfamily, which encompasses (in addition to Rho) Rac, Cdc42, RhoD, Rnd/RhoE, RhoG, and TC10. These low molecular mass GT-Pases are involved in the regulation of the actin cytoskeleton, membrane trafficking, cell cycle progression, cell transformation, and apoptosis. The function of the GTPases is tightly governed by regulatory proteins such as the exchange factors, the GTPase-activating proteins, and the guanine nucleotide dissociation inhibitors. Downstream signaling is mediated through interaction with effector proteins, which are serine/threonine-kinases (Rho kinases, protein kinase N), lipid kinases (phosphatidyl inositol kinases), phospholipases (phospholipase D), and adapter proteins (rhoetkin, rhophilin, p140Dia, myosin binding subunit) (13–16). The finding, that C3-catalyzed ADP-ribosylation of RhoA in intact cells causes depolymerization of the actin filaments, led to the notion that ADP-ribosylation at Asn^41 inactivates Rho. ADP-ribosylation has no significant influence on nucleotide exchange, intrinsic and GTPase-activating protein-stimulated GTPase activity but clearly decreases the exchange activity of Lbc (17–19). Furthermore, the guanine nucleotide dissociation inhibitor-driven cycling between cytosol and membranes is blocked leading to sequestration of ADP-ribosylated Rho in the inactive guanine nucleotide dissociation inhibitor complex. This sequestration seems to be an additional important functional consequence finally resulting in the inactivation of Rho.

The Rho proteins are also targets for other bacterial toxins. The *Clostridium difficile* toxins A and B glucosylate Rho at Thr^37 but also modify Rac and Cdc42 (21, 22). A comparable substrate specificity exhibits the cytotoxic necrotizing factor from *Escherichia coli*, which catalyzes deamidation of the residue Glu^63 of RhoA (23, 24). Among the Rho-modifying toxins, the C3-like exoenzymes exhibit the most confined substrate specificity to exclusively ADP-ribosylate RhoA, -B, and -C. The Rho-related GTPases Rac and Cdc42 also harbor the acceptor amino acid asparagine (Asn^39 in Rac and Cdc42) and are highly homologous in the vicinity of the acceptor amino acid but are not substrates for C3. We studied, therefore, which amino acids of Rho define the substrate specificity for C3 and whether it is possible to construct a mutant Rac that is a substrate for C3.

**EXPERIMENTAL PROCEDURES**

Materials and Chemicals—RhoA, Rac1, and the mutant forms were purified as GST-fusion proteins from *E. coli* followed by thrombin cleavage. Thrombin was removed by precipitation with benzamidine.

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2 The abbreviations used are: GST, glutathione S-transferase; PCR, polymerase chain reaction; SOE, splicing by overlapping extension; GTP^S, guanosine 5'-3'-O-(thiotriphosphate); Gpp(NH)p, guanylyl-5'-yImidodiphosphate; PAGE, polyacrylamide gel electrophoresis.
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Sepharose. Oligonucleotides were obtained from MWG (Ebersberg, Germany), the pGEX-2T vector and the glutathione S-transferase gene fusion system were from Amersham Pharmacia Biotech, the Quickchange kit was from Stratagene (Heidelberg, Germany), and the restriction enzymes were from NEB (Schwalbach, Germany). The secondary anti-rabbit peroxidase-conjugated antibody was from Roche (Mannheim, Germany), and anti-mouse IgG (diluted 1:1000) was from Dianova (Hamburg, Germany). The anti-rabbit IgG conjugated with horseradish peroxidase was from BioRad Laboratories (Hercules, CA). Other chemicals were from commercial sources. C. botulinum exoenzyme C3 was prepared as described (25).

**Construction of Mutant Rac1 or RhoA Proteins**—The RhoA or Rac1 mutants were constructed by site-directed mutagenesis using the pGEX2T-wild type human RhoA or the pGEX2T-wild type human Rac1 vector as templates and the respective oligonucleotides using the Quickchange kit according to the manufacturer’s instructions.

The PCR reactions were carried out with Phusion polymerase according to the manual from Stratagene or with Taq-DNA polymerase (Roche Molecular Biochemicals) and the TOPO-TA vector system from Invitrogen (Leek, The Netherlands) in a Gene Amp 2400 PCR system from Perkin-Elmer. DNA was sequenced with the cycle sequencing ready reaction kit (ABI PRISM) from Perkin-Elmer to verify the correct mutations.

**Construction of RhoA90Rac1 Chimeras**—This chimeras was created using the pGEX2T vectors mentioned above and a splitting by overlapping extension (“SOE”) PCR reaction (26). The primers are: SOE RhoA, 5'-gag att tta aaa tga atc agg ggt gag gga and SOE RhoA C, 5’- gaa aat gta aga tga tgc GC and SOE Rac1, 5’-gag agc cct gat tca ttt gaa aat gtc gtt gc and Rac1 N, 5’-cag tgc cag cga tc and a representative kit of PCR products were separated by agarose gel electrophoresis, purified, and put in a third PCR as template for each other. As a result, a 1.8-kilobase fragment was obtained and ligated in the TA vector system. Sequential digestion with BstBI and PstI resulted in mobilization of the PCR product, which can be ligated directly into a pGEX2T, cut with the same enzymes. The resulted plasmid was transformed in *E. coli* XL-1 Blue supercompetent cells (Stratagene). Binding of N-methylanthraniloyl-GDP was used to prove structural and functional integrity of the mutant Rho and Rac proteins. The GTPases (0.5 μM), dissolved in buffer C (10 mM Tris-HCl, pH 7.5, 25 mM MgCl₂, 150 mM NaCl) were warmed to 37°C, and the nucleotide exchange was initiated by the addition of 5 mM MgCl₂ at 37°C for 3 h. Samples were filtered through a 10-kDa Centricon, and the protein-free flow through was separated by TLC on Silica Gel 60 F254 (Merck) with 66% 2-propanol and 0.33% ammonium sulfate. The amount of hydrolyzed [³²P]NAD was calculated from PhosphorImager data.

**RESULTS**

To test whether the complete structure of Rho or only subdomains define the substrate recognition by C3 exoenzyme, chimeras between RhoA and Rac1 were constructed (Fig. 1) and tested for ADP-ribosylation. Only when the N-terminal part was Rho-like, the chimeras were modified by C3 (Fig. 1). Thus, the minimal part, which makes the difference between Rac and Rho with respect to ADP-ribosylation, is determined by amino acids 1–90. The non-ADP-ribosylatable chimeras were native in structure as tested by nucleotide binding (data not shown) except the Rho73Rac chimera, which was therefore excluded.

Sequence comparison of the N-terminal 90 amino acids of RhoA with Rac1 revealed several differences (Fig. 2). The basic stretch at the N terminus (position RhoArg⁵-Lys⁶), which is absent in Rac, and the acidic residues RacArg⁴⁷-Lys⁵⁴, RacAsp⁷⁶, RacAsp⁸⁷, and RacAsp⁹⁰, which are not acidic in Rac, were inserted and exchanged, respectively. Mutant Rac1 proteins possessing the respective amino acid(s) of RhoA were then tested for ADP-ribosylation as depicted in Table I. Only when the N-terminal part was Rho-like, the chimeras were modified by C3 (Fig. 1). Therefore, combinations of those mutations identified to affect ADP-ribosylation were tested. The basic insert (residues RacArg⁴⁷-Lys⁵⁴) together with RacGlu⁴⁷ and RacGlu⁵⁴ led to a significant increase in ADP-ribosylation, however, only to a minor degree. Therefore, combinations of those mutations identified to affect ADP-ribosylation were tested. The basic insert (residues RacArg⁴⁷-Lys⁵⁴) together with RacGlu⁴⁷ and RacGlu⁵⁴ led to a significant increase in ADP-ribosylation of this mutant Rac (Table I; note that the insert of the two basic residues changes the numbering of the Rac1 residues). To compare the kinetics of the ADP-ribosylation reaction between wild type RhoA and Rac⁴⁷⁸⁷⁵⁴-Glu⁴⁷⁵⁴, the concentration of C3 was decreased from 1 nM to 0.1 nM (4 nM) to extend the linear phase. Surprisingly, the mutant Rac protein (Rac⁴⁷⁸⁷⁵⁴-Glu⁴⁷⁵⁴-Glu⁴⁷⁵⁴) was only faintly modified, indicating that the so far identified residues only partially contributed to the substrate recognition by C3 (Fig. 3). As can be deduced from the crystal structure of RhoA, Asn⁴¹ is surrounded by solvent-exposed lipophilic residues, such as...
Val^{38}, Phe^{39}, Val^{43}, and Trp^{58} (27). Only Val^{43} is different in Rac, the equivalent position is taken by a hydrophilic serine. The side-chain of Asn^{41} interacts with Trp^{58} and with Glu^{40}, the latter one is an Asp residue in Rac^{(RacAsp^{38})}. The exchange of this two residues in Rho (Glu^{40} → Asp, Val^{43} → Ser) to the respective amino acids of Rac, almost completely inhibited

![Fig. 2. Alignment of the N-terminal part of RhoA and Rac1. Amino acids 1–90 of RhoA^{WT} and Rac1^{WT} are aligned. Boxes show the differences, and arrows highlight the mutated amino acids in Rac1. The numbers below or above indicate the amino acid position in Rac1 or RhoA, respectively.](#)

![Fig. 3. Time course of ADP-ribosylation of RhoA^{WT}, Rac1^{WT}, and various Rac1 mutants.](#)

![Fig. 4. A, Glu^{38} and Val^{43} of Rho are essential for the C3-catalyzed ADP-ribosylation.](#)
The residues identified to promote ADP-ribosylation by C3 were tested whether they participate in substrate recognition, i.e. binding to C3. To this end, the binding of multiple mutant Rho and Rac proteins to C3 was studied by applying a precipitation assay with immobilized mutant GST fusion proteins. Preliminary experiments showed that C3 binding was different from nonspecific binding. As illustrated in Fig. 5A, C3 bound to wild type RhoA but not to wild type Rac whose binding was comparable to control signal. The binding of the poor ADP-ribosylatable RacArg5-Lys6-Glu47-Glu54 and RacGlu38-Val41 was comparable to C3 binding to RhoA. Furthermore, increasing concentrations of C3 resulted in increased binding but not increased nonspecific binding. The amount of RhoA-bound C3 was about 25% (Fig. 5B). As shown in Fig. 5C, RacArg5-Lys6-Glu47-Glu54 and RacGlu38-Val41 exhibited the same binding as the full substrate mutant Rac (RacArg5-Lys6-Glu40-Val43-Glu47-Glu54). Thus, the combination of all mutations, which contributed to ADP-ribosylation, did not further increase binding to C3. Consistent with this finding was that the exchange of RhoGlu40 and RhoVal43 did not alter the binding to C3. The exchange of RhoTrp58, which abolished ADP-ribosylation had no effect on C3 binding. Thus, there are two types of binding and only the combination makes Rac1 a substrate. It is conceivable that the residues involved in mere binding to C3 also participate in the correct formation of the ternary complex, consisting of RhoA, C3, and NAD\(^+\). Especially, the residues RhoGlu40 and RhoVal43, which are adjacent to the acceptor residue Asn41, could be involved in the correct positioning of the catalytic site of C3 in respect to the acceptor amino acid Asn41. To prove this working hypothesis, we made use of the fact that the NAD glycohydrolase activity of C3 was increased by mutant Rho and deficient in the acceptor amino acid Asn41 (RhoIle41) (Fig. 6). This observation seems to be because of the fact that the formation of the ternary complex increases the rate of glycohydrolase probably by decreasing the \(k_m\) for NAD. Although both RhoPhe39 and RhoAsp40-Ser43 bound to C3 and were non-ADP-ribosylatable, only RhoPhe39 was capable of increasing the glycohydrolase activity, whereas RhoAsp40-Ser43 even blocked it (Fig. 6). The inhibitory effect of RhoAsp40-Ser43 might be because of an incorrect interaction of C3 with RhoA not allowing the correct binding of NAD\(^+\).

The acceptor amino acid RhoAsn41 is located in the effector loop (switch I) of RhoA, which undergoes conformational changes upon GTP binding. To test whether nucleotide occupancy affects the accessibility of the amide moiety of Asn41, the nucleotide dependence of the ADP-ribosylation reaction was studied. To exclude GTPase activity to form GDP during the ADP-ribosylation reaction, the nonhydrolyzable GTP analogues Gpp(NH)p and GTP\(_\gamma\)S were used. Fig. 7 shows the linear phase of the ADP-ribosylation reaction of RhoA loaded with either GDP, Gpp(NH)p, or GTP\(_\gamma\)S. The rate of ADP-ribosylation of RhoA-GDP was about five times faster than that of RhoA-Gpp(NH)p/GTP\(_\gamma\)S. The total amount of ADP-ribosyl incorporation, however, was not changed (Fig. 7, inset). Thus, the nucleotide occupancy had only minor effects on the kinetics of the transferase reaction.

DISCUSSION

The C3-like ADP-ribosyltransferases are characterized by a very selective substrate specificity to exclusively modify the Rho isoforms A, B, and C, which possess a homology of about 90%. This specificity is striking, because other Rho-modifying bacterial toxins exhibit less specificity. Furthermore, this speci-
licity of C3.

However, only the crystal structure of RhoA (27). In that study, the authors point to the extent nor the time course of ADP-ribosylation were resolved on TLC, and cleaved $^{32}$P-ADP-ribose was determined by phosphoimaging.

...ADP-ribosylation; samples from the linear phase of the reaction described under "Experimental Procedures" followed by C3-catalyzed hydrophilic serine (RacSer41). Indeed, RhoVal43 is important for substrate recognition by C3. From the binding experiments of the wild type and mutant RhoA and Rac1, respectively, it became clear that the basic residues at the N terminus together with acidic residues (RhoGlu47 and RhoGlu54) and the RhoGlu40/RhoVal43 residues are independently responsible for the mere binding to C3. The combination, i.e. the generation of the 6-fold mutant, does not increase binding. The residues involved in binding of Rho to C3 are exclusively located in the N-terminal part; thus, it is unlikely that the C-terminal part of RhoA contributes to the binding.

However, the residues RhoGlu40/RhoVal43 have a profound function in the transfer of the ADP-ribose moiety because their exchange results in abolished ADP-ribosylation. In the case of the NAD-glycohydrolase reaction, a binary complex is formed between C3 and NAD$^+$, and the rate of reaction is much slower (factor of 100) compared with the transferase reaction. The presence of non-ADP-ribosylatable Rho (RhoAsp38) increases the glycohydrolase activity most likely by increasing the affinity of NAD to the now existing ternary complex. In contrast, mutant Rho lacking Glu40 and Val43, which still binds to C3, does block the NAD-glycohydrolase activity, most likely through its inability to form the correct ternary complex. Taken together, the residues Glu40 and Val43 are directly involved in the correct formation of the ternary complex formed by the exoenzyme C3, the protein substrate RhoA, and the co-substrate NAD$^+$.

The acceptor amino acid for ADP-ribosylation, Asn41, resides in the effector loop, as it can be concluded from the effects of partial loss of function mutants. The exchange of RhoThr42, the residue adjacent to Asn41, to Cys decreases PLD1 activation, whereas mutation of Asn41 has no effect on stimulation (28). Also the exchange of Glu40, the residue just before Asn41, inhibits effector activation, namely the RhoA stimulation of Rho kinase (ROK) (29). Switch I undergoes conformational changes during nucleotide exchange but the solvent accessibility of the side chain of Asn41 does not change, as can be deduced from the crystal structures of Rho-GTP and Rho-GDP (27, 30). The nucleotide-dependent time course of the ADP-ribosylation revealed that Rho-GDP is the optimal substrate and that binding of the GTP analogues Gpp(NH)p or GTP$^+$ decreases the rate of reaction by a factor of five but does not change the total amount of ADP-ribosylation. Thus, conformational changes induced by nucleotide binding have only a minor effect on C3-catalyzed ADP-ribosylation. However, this effect is only gradually compared with that of monoglycosylation of RhoA at Thr37 by Clostridium novyi a-toxin and of H-Ras at Thr20 by the Clostridium sordellii lethal toxin (31, 32). Because the hydroxyl group of RhoThr37 (RasThr35) points to the inner core of
the protein (20, 33). RhoA-GTP (Ras-GTP) is resistant to glucosylation. Therefore, the GDP form of RhoA is exclusively modified. Because the nucleotide occupancy has only effects on the kinetics but not the total amount of ADP-ribosylation, C3-catalyzed ADP-ribosylation is in fact not a reliable marker to determine the inactive fraction of Rho in cells.

In conclusion, it turned out that the basic stretch and at least three acidic amino acids are involved in the binding to C3. The N-terminal part of Rho has more acidic amino acids at the surface of the molecule than Rac. Thus, the specificity of Rho to serve as substrate for C3 is determined by several amino acids distributed over the N-terminal part of Rho. It is not possible to generate mutant Rac to be substrate of C3 by single or double exchange of amino acids. At least six residues are responsible for the interaction with C3 and thus define the selective interaction with Rho.

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