A Novel C3-like ADP-ribosyltransferase from *Staphylococcus aureus* Modifying RhoE and Rnd3*

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Clostridium botulinum C3 is the prototype of the family of the C3-like transferases that ADP-ribosylate exclusively RhoA, -B and -C. The ADP-ribose at Asn-41 results in functional inactivation of Rho reflected by disaggregation of the actin cytoskeleton. We report on a new C3-like transferase produced by a pathogenic *Staphylococcus aureus* strain. The transferase designated C3\(^{Stau}\) was cloned from the genomic DNA. At the amino acid level, C3\(^{Stau}\) revealed an identity of 35% to C3 from *C. botulinum* and *Clostridium limosum* exoenzyme, respectively, and of 78% to EDIN from *S. aureus*. In addition to RhoA, which is the target of the other C3-like transferases, C3\(^{Stau}\) modified RhoE and Rnd3. RhoE was ADP-ribosylated at Asn-44, which is equivalent to Asn-41 of RhoA. RhoE and Rnd3 are members of the Rho subfamily, which are deficient in intrinsic GTPase activity and possess a Rho antagonistic cell function. The protein substrate specificity found with recombinant Rho proteins was corroborated by expression of RhoE in *Xenopus laevis* oocytes showing that RhoE was also modified in vivo by C3\(^{Stau}\) but not by C3 from *C. botulinum*. The poor cell accessibility of C3\(^{Stau}\) was overcome by generation of a chimeric toxin recruiting the cell entry machinery of *C. botulinum* C2 toxin. The chimeric C3\(^{Stau}\) caused the same morphological and cytoskeletal changes as the chimeric *C. botulinum* C3. C3\(^{Stau}\) is a new member of the family of the C3-like transferases but is also the prototype of a subgroup of RhoE/Rnd modifying transferases.

Various bacterial protein toxins interfere with eukaryotic cell functions by catalyzing a posttranslational modification of essential cellular regulator proteins such as ADP-ribosylation of the Rho proteins by C3-like toxin. *Clostridium botulinum* exoenzyme C3 is the prototype of a family encompassing exoenzymes from *Clostridium limosum* and *Bacillus cereus* and from *Staphylococcus aureus* (EDIN)\(^1\) (1–4). The members of this family are similar in structure and homologous to each other. They are single-chained ADP-ribosyltransferases with a molecular mass of ~25 kDa. The C3-like transferases are in fact mere exoenzymes devoid of the cell entry apparatus harbored by other toxins, and they are thought to enter cells by nonspecific pinocytosis. C3 catalyzes ADP-ribosylation of the RhoA, -B and -C subtypes but not of other members of Rho and Ras subfamilies (3–5). Only in the presence of the detergent sodium dodecyl sulfate, the Rac protein is a poor substrate (4). The ADP-ribose moiety is transferred from NAD\(^+\) to the acceptor amino acid Asn-41 and is linked N-glycosidically to the amide group of the carboxylate side chain of Asn-41 (6).

The Rho proteins belong to the Ras superfamily of low molecular mass GTPases, which are the major regulators of the actin cytoskeleton, but they are also involved in cell cycle progression, transcriptional activity, and in cooperation with Ras in cell transformation (7–9). Because ADP-ribosylation of Rho in intact cells results in disaggregation of the actin cytoskeleton, ADP-ribosylation has been classified as an inactivating modification. The molecular basis of the inactivation is the decreased interaction of Rho with the exchange factors, which promote activation (10), and the sequestration by the negative regulator guanine nucleotide exchange factor.\(^2\) Although C3 induces the breakdown of the actin cytoskeleton, an effect that can be easily monitored, it is now clear that C3-catalyzed ADP-ribosylation inactivates all Rho functions (for reviews see Refs. 12–14).

Because of its confined protein substrate specificity, C3 has been advanced to a widely used tool in cell biology to selectively turn off cellular Rho functions. The poor cell accessibility of C3 has been overcome by the creation of chimeric C3 toxins using the cell entry apparatus of other toxins (15, 16). We report here on a novel C3-like transferase produced by a pathogenic *S. aureus* strain that ADP-ribosylates RhoE/Rnd3 subtype proteins in addition to RhoA, -B and -C.

**EXPERIMENTAL PROCEDURES**

**Materials and Chemicals**—Culture supernatants were subjected to purification using the FPLC System, an anion exchange MonoQ column, and a cation exchange MonoS column from Amersham Pharmacia Biotech. Oligonucleotides were obtained from Mannfred Weichertsgartner (Ebersberg, Germany), PCR was carried out using the Gene Amp 2400 System from PerkinElmer Life Sciences, and DNA sequencing was carried out with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit from PerkinElmer Life Sciences. The Topo-TA-vector system was from Invitrogen (Groningen, The Netherlands), the pGEX2T vector system from Amersham Pharmacia Biotech, and the QuickChange Kit was from Stratagene (Heidelberg, Germany). Restriction

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1 The abbreviations used are: EDIN, epidermal differentiation inhibitor from *S. aureus* strain E1; PCR, polymerase chain reaction; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; C3\(^{Stau}\), C. botulinum exoenzyme C3; C3\(^{lim}\), C. limosum ADP-ribosyltransferase; C3\(^{Stau}\), ADP-ribosyltransferase from *S. aureus* strain HM16.

2 H. Genth, M. Schmidt, H. Barth, K. Aktories, and I. Just, submitted for publication.
enzymes and T4-DNA ligase were from New England Biolabs. C. botulinum C3 exoenzyme was purified as described (17). All other chemicals were from commercial sources.

RhoE, as described by the group of Settleman and co-workers (18), RhoA, Rac1, CDC42, and TC10 were purified as glutathione S-transferase fusion proteins, and RhoD was expressed as maltose-binding protein in Escherichia coli. Rnd3 was cloned using the RhoE construct from Settleman and co-workers (18) and an extension consisting of the published nucleotide sequence encoding for an N-terminal 15-amino acid fragment from Rnd3 (22). In some cases, the GST portion was cleaved off with thrombin (100 μg/ml), which was removed by precipitation with benzamidine-Sepharose beads (Amersham Pharmacia Biotech).

**Purification—** S. aureus strain HMI6 was a clinical isolate from a patient with postoperative infection. The identity of this isolate was confirmed in the laboratory by routine tests. For purification of the ADP-ribosyltransferase, bacteria were grown in LB medium at 37 °C overnight. After centrifugation at 4 °C, ammonium sulfate was added to a concentration of 70% to precipitate proteins from the culture supernatant. RhoD was expressed as maltose-binding protein in 50 mM HEPES, pH 7.0, and dialyzed against the same buffer overnight to remove the salt. The solution was added onto the MonoQ column using a 10-ml super loop, and the flow through was collected, dialyzed against a buffer containing 50 mM HEPES, pH 6.0, and loaded onto the MonoQ column. The transferrase was eluted with a linear gradient at 0.3 M NaCl in 50 mM HEPES, pH 7.0, and 0.1 M NaCl.

**ADP-ribosylation Reaction—** Recombinant RhoA, RhoE/Rnd3, and other GTPases (2 μg) were ADP-ribosylated in a buffer containing 50 mM HEPES, pH 7.3, 2 mM MgCl₂, 20 μM [adenylate-32P]NAD, and 100 μg/ml bovine serum albumin for up to 4 h at 37 °C. The total volume was 25 μl. The recombinant toxin was applied in a concentration of 1 mM or as indicated.

**SDS-PAGE—** SDS-polyacrylamide gel electrophoresis was performed according to the methods of Laemmli (19). Gels were stained with Coomassie Brilliant Blue R-250, dried, and further analyzed by the PhosphorImager SI from Molecular Dynamics.

**Amino Acid Sequencing Analysis—** The eluted fraction from the MonoS column containing the transferrase was separated by SDS-PAGE, transferred onto polyvinylidene difluoride membrane (Amersham Pharmacia Biotech), and visualized with Amido Black. N-terminal amino acid sequencing was performed on an excised band using an Applied Biosystems 447A pulse-liquid protein sequencer.

**Amplification from Genomic DNA—** Genomic DNA from strain S. aureus HMI6 was prepared by standard methods. Primers for amplification of the HMI6 gene were designed according to the determined N-terminal amino acid sequence and in consideration of the staphylococci codon usage. For the 3' end, the primer was designed according to a region 100 base pairs downstream of the in frame stop codon in the cloned EDIN sequence (2). PCR was performed under the following conditions: 500 ng of template, 2.5 mM of each dNTP, 5 μl of 10-fold concentrated Mg²⁺-free buffer, different concentrations of MgCl₂, and variant units of Taq polymerase (New England Biolabs). The primers were: HMI6-N, 5' AGA TCT GCC GAC ACT AAA AAT TTT ACA G 3'; and HMI6-C, 5' GGA TCC TAA GTC TTA ACC GTA TTT TTA G 3'. PCR products were separated by gel electrophoresis, purified, ligated into the TOPO-TA vector, and sequenced. For mobilization of the C3Stau gene, the HMI6-5' end primer and a primer corresponding to the 3' end of C3Stau were used. The primers were flanked by a 5' BglII and a 3' BamHI site, which were used for the ligation of the C3Stau gene into the pGEX vector. The amino acid sequence of C3Stau was available in the GenBankTM/EBI database under accession number A2277173.

**Construction of Recombinant C3Stau Transferrase—** For expression of the transferrase, bacteria were grown overnight in LB media in the presence of 100 μg/ml of ampicillin, followed by inoculation in fresh media. At an A₆₀₀ of 0.8, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.2 mM. After incubation for 6 h at 37 °C, bacteria were pelleted, resuspended in lysis buffer containing 50 mM HEPES, pH 7.3, 2 mM MgCl₂, and 1 mM phenylmethylsulfonyl fluoride with a French Press (SLM Aminco). Debris was removed by centrifugation for 15 min at 15,000 × g at 4 °C, and GST fusion protein was purified from supernatant with glutathione-Sepharose beads. The GST carrier was cleaved with thrombin followed by its removal with benzamidine beads.

**Preparation of Oocyte Lysates Overexpressing Human RhoE—** Human RhoE cRNA was transcribed in vitro using the RhoE-pNK2 as template and injected into defolliculated Xenopus laevis oocytes. After 24 h at 19 °C, lysates were prepared as described from cRNA-infected cells and noninjected cells (20).

**RESULTS**

Several clinical isolates of S. aureus were screened for C3-like activity. An isolate designated HMI6 was identified to possess ADP-ribosyltransferase activity, which modified recombinant RhoA. The transferrase was purified from the culture supernatant by ammonium sulfate precipitation and ion exchange chromatography. The partially purified protein was excised from SDS-polyacrylamide gel and N-terminally sequenced. Twenty-three amino acids of the N terminus were identified revealing an identity of 78% (18 of 23 amino acids) with the ADP-ribosyltransferase from S. aureus. Several recombinant proteins were expressed, and RhoD was expressed as maltose-binding protein in 50 mM HEPES, pH 7.0, and loaded onto the MonoQ column separation. The transferrase was eluted with a linear gradient at 0.3 M NaCl in 50 mM HEPES, pH 7.0, and 0.1 M NaCl.

**Construction of the C2IN-C3Stau Fusion Toxin—** The fusion toxin containing the N-terminal 225 amino acids from C. botulinum C2 toxin and the full-length C3Stau was constructed using the 5' BamHI site and the 3' BamHI site flanking the toxin gene. Construction, expression, and purification of GST fusion protein were carried out as described (16).

**Cytotoxic Assay—** NIH3T3 and KB cells were grown to subconfluency at 37 °C in Dulbecco's minimum essential medium containing 10% fetal calf serum, 2 mM glutamine, 100 units penicillin/ml, and 100 μg/ml streptomycin at 5% CO₂. For cytotoxic assays, subconfluent cells in 24-well plates containing coverslips were treated for 3 h with 200 ng/ml activated C2II toxin, C2IN-C3Stau alone (100 ng/ml), or with 200 ng/ml activated C2II and C2IN-C3Stau. As a control, cells were treated with 200 ng/ml activated C2II and 100 ng/ml C2IN-C3 (limosum) for the same time. Cells growing on coverslips were washed twice with phosphate-buffered saline and fixed with 4% paraformaldehyde and 0.1% Triton X-100 in phosphate-buffered saline for 30 min. Actin was stained with phalloidin-rhodamine (600 ng/ml) and the coverslips were mounted in Moviol.

**Alignment of the N-terminal amino acids of the ADP-ribosyltransferase C3Stau from S. aureus strain HMI6 with EDIN from S. aureus strain E1.** Partially purified C3Stau was N-terminally sequenced. The amino acids marked in white are those different in C3Stau.

[Insert figure 1. Alignment of the N-terminal amino acids of the ADP-ribosyltransferase C3Stau from S. aureus strain HMI6 with EDIN from S. aureus strain E1. Partially purified C3Stau was N-terminally sequenced. The amino acids marked in white are those different in C3Stau.]

The coding gene for C3Stau was amplified from the genomic DNA by PCR. The 5' primers were deduced from the identified N-terminal amino acid sequence, and the basis for the 3' primers was the noncoding region EDIN, the sequence of which is known. Indeed, one major PCR product was obtained. From this product, the C3Stau gene was further cloned. The determined sequence is presented in Fig. 2, and the alignment with the sequences of EDIN, C3bot, and C3lim is given in Fig. 3. The molecular mass of C3Stau was calculated as 23,640 Da, and the theoretical isoelectric point was 9.4. The sequence comparison revealed an identity of 78% (18 of 23 amino acids) with the C3Stau transfectase from S. aureus. The ADP-ribosyltransferase from S. aureus was designated C3Stau to make a distinction from C3bot (C. botulinum), C3lim (C. limosum), and C3Stau (B. cereus).

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To test the protein substrate specificity, recombinant Rho subfamily proteins (RhoA, Rac1, Cdc42, RhoD, RhoE, TC10, and RhoN) were incubated with C3Stau in the presence of [32P]NAD. As shown in Fig. 5A, RhoA was modified by C3Stau. Surprisingly, RhoE was modified by C3Stau but not by C3bot. The new substrate specificity of C3Stau was corroborated by showing that Rnd3 was also a substrate.
Rnd3 is an isoform of RhoE possessing an N-terminal 15-amino acid extension. The time course revealed that RhoE/Rnd3 was ADP-ribosylated more slowly by C3Stau compared with RhoA, and the linear phase of ADP-ribosylation lasted for hours. C3bot, in contrast, did not catalyze any incorporation of ADP-ribose into RhoE/Rnd3 (Fig. 5B). RhoE and Rnd3 have been identified recently as GTPase-deficient low molecular mass GTP-binding protein, which has preferentially bound GTP (18, 21, 22). Because RhoA in the GDP-bound but not in the GTP-bound form is the preferred substrate for C3bot (23), RhoE was artificially loaded with GDP and tested for ADP-ribosylation. However, the nucleotide occupancy did not change the kinetics of modification (data not shown). Thus, for unknown reasons, RhoE/Rnd3 in contrast to RhoA was ADP-ribosylated with slow velocity.

Sequential ADP-ribosylation of recombinant RhoA, i.e. first with C3bot followed by C3Stau (and vice versa), indicated that both transferases linked the ADP-ribose to Asn-41 of RhoA (data not shown). This finding was confirmed by using the mutant RhoA<sub>Asn41Ile</sub>, which was not a substrate for C3Stau and C3bot (Fig. 5C). Asn-41 is equivalent to Asn-44 in RhoE. Its exchange to Ile (RhoE<sub>Asn44Ile</sub>) completely prevented ADP-ribosylation by C3Stau, indicating that Asn-44 is the acceptor amino acid in RhoE (Fig. 5C).

Another approach to check whether RhoE was an in vivo substrate for C3Stau was the expression of RhoE in oocytes from <i>X. laevis</i>. In lysates from noninjected control oocytes, C3Stau and C3bot [32P]ADP-ribosylated only RhoA (one band) (Fig. 6B). However, in lysates from oocytes expressing the RhoE protein, C3Stau ADP-ribosylated two polypeptides (double band), whereas C3bot modified only one single band (Fig. 6B). Thus, cellular RhoE was a substrate for C3 Stau, corroborating the findings with recombinant Rho GTPases.

Exoenzymes C3bot and C3lim are cytotoxic to cultured cell lines to induce disaggregation of actin filaments but only when applied at micromolar concentrations (13). The same property was true for C3Stau (data not shown). To overcome this limita-
tion, a chimeric C3Stau was constructed analogous to that construct of C3lim with C. botulinum C2 toxin (16). C3Stau was fused to the enzymatically deficient C2I component, and the cell entry was mediated by the receptor binding component C2II from C. botulinum. C2IN-C3Stau was as nontoxic to cells as C3Stau was, but in the presence of C2II C2IN-C3Stau induced the typical C3-like morphology and cytoskeletal changes (Fig. 7A). The differential ADP-ribosylation of lysates from intoxicated cells clearly demonstrated the in vivo ADP-ribosylation of cellular RhoA (Fig. 7B).

**DISCUSSION**

The first exoenzyme that was identified to ADP-ribosylate the Rho protein was exoenzyme C3 from C. botulinum. Because several isoforms of C3, all produced by C. botulinum types C and D, were identified, it was initially thought that C3 was associated with or even part of the botulinum neurotoxins. However, the later identification of homologues of C3 produced by C. limosum, B. cereus, and S. aureus, which definitely do not harbor genes for neurotoxins, proved that C3 and C3-like exoenzymes are independent from neurotoxins (24). All the C3 homologues are single chain proteins that are released from the bacteria and are, therefore, to be classified as exoenzymes. Compared with bacterial protein toxins such as pertussis toxin, cholera toxin, or *Pseudomonas* exotoxin A, the C3 homologues are devoid of a cell entry apparatus and seem to enter cells by nonspecific pinocytosis (25). Their function as virulence factors is unclear, but there is a report

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**Fig. 5.** Protein substrate specificity of C3Stau. **A,** protein substrate specificity of C3Stau compared with that of C3bot. The recombinant low molecular mass GTPases of the Rho subfamily, RhoA, Rac1, RhoE, Rnd3, Cdc42, TC10, RhoD, and RhoN were [32P]ADP-ribosylated by C3bot (upper lane) and by C3Stau (lower lane); the GTPases were separated by SDS-PAGE and subsequently analyzed by phosphorimaging (shown). **B,** time- and concentration-dependent ADP-ribosylation of RhoE (solid line) and Rnd3 (dotted line). Recombinant proteins were [32P]ADP-ribosylated by C3bot and by C3Stau. After the indicated time points, samples were analyzed by SDS-PAGE and were evaluated by phosphorimaging. The amount of incorporated [32P]ADP-ribose was given in moles of ADP-ribose per mole of RhoE/Rnd3. **C,** acceptor amino acid of ADP-ribosylation. RhoAAsn41 and equivalent RhoEAsn44 were changed to isoleucine (Ile). Wild-type (WT) and mutated RhoA/RhoE were [32P]ADP-ribosylated by C3Stau and C3bot. PhosphorImager data are shown.

**Fig. 6.** RhoE is the intracellular substrate of C3Stau. **A,** sequential ADP-ribosylation of rat brain membranes with C3Stau and C3bot. The membrane fractions from rat brain were ADP-ribosylated by C3bot in the presence of unlabeled NAD. After washing, the membranes were divided and [32P]ADP-ribosylated by C3Stau and C3bot, respectively. Samples were run on SDS-PAGE and analyzed by a PhosphorImager. **B,** the lysates of *X. laevis* oocytes were injected with RhoE cRNA, and noninjected controls were [32P]ADP-ribosylated by C3Stau or C3bot. The PhosphorImager data of the SDS-PAGE are shown.
that the C3 homologue EDIN from S. aureus inhibits differentiation and induces hyperplasia of epidermis (26). The C3 homologue C3Stau, presented in this study, is also produced by a pathogenic S. aureus strain. C3Stau shows 78% identity and 90% homology to EDIN but only 35% identity to C3 isoforms and the exoenzyme from C. limosum.

The identification of C3 exoenzyme is tightly linked to the elucidation of C3-like Rho proteins (13, 24). The reason for this is the remarkable substrate specificity of C3Stau, which ADP-ribosylates only the subtypes RhoA, -B, and -C but not RhoE/Rnd3. RhoA, -B, and -C, the recently identified Rho subfamily members, have a remarkable substrate specificity of their ADP-ribosylation. The lysates of treated and nontreated NIH3T3 cells were [32P]ADP-ribosylated with C3Stau. The proteins were resolved on SDS-PAGE and analyzed by phosphorimaging.

ADP-ribosyltransferase from S. aureus

RhoE/Rnd3 belongs to a new branch of the Rho subfamily, the Rnd proteins (22). The members Rnd1, Rnd2, and Rnd3, the last one being nearly identical with RhoE, are about 50% identical with RhoA, but they show a remarkable functional difference to RhoA. They are deficient in intrinsic and GTPase-activating protein-stimulated GTPase activity (18, 21, 22). Furthermore, RhoE/Rnd3 has the opposite function of RhoA and can be classified as a functional RhoA antagonist (21, 22). How RhoE/Rnd3 is regulated is far from understood, possibly by expression or by subcellular translocation.

The initial step of RhoA activation, the interaction with guanine nucleotide exchange factor Lbc, is blocked by ADP-ribosylation (10). Based on this finding it is conceivable that RhoE/Rnd3 acts by sequestering exchange factors, thereby preventing activation of RhoA. ADP-ribosylation might inhibit binding of RhoE/Rnd3 to the exchange factors, thereby allowing the normal activation cascade of RhoA.

In the case of C3bot and C3lim, inactivation of RhoA by ADP-ribosylation allows RhoE/Rnd3 to act, thereby inducing disaggregation of the actin cytoskeleton. C3Stau, however, inactivates both RhoA and its antagonist RhoE/Rnd3. Thus, one expects fewer morphological effects for C3Stau than for C3bot. However, the experimental data do not support this notion. The major reason for this might be the low expression of RhoE/Rnd3 in all tissues tested, which does not allow a direct functional antagonism to RhoA (22).

C3Stau belongs to the family of C3-like transferases because of its homology and its ability to ADP-ribosylate RhoA. However, it is also the prototype of a novel subfamily of the C3-like transferases because of its extended protein substrate specificity, modifying RhoE and Rnd3 in addition to RhoA. C3Stau and EDIN are produced by pathogenic S. aureus strains. S. aureus bacteria are reported to escape endosomes after phagocytosis by endothelial cells and to exist freely in the cytoplasm (11, 27). Based on this scenario, C3Stau as well as EDIN are released inside the cytoplasm and can immediately reach their targets RhoA, RhoE, and Rnd3. Under these conditions they in fact do not need any cell entry machinery.

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Fig. 7. Cytotoxic effects of chimeric C2IN-C3Stau on NIH3T3 cells. A, cells were treated with the single components and their combinations for 3 h followed by staining with rhodamine-phalloidin. a, control cells, b, C2IN-C3Stau (100 ng/ml), c, C2IN-C3bot (100 ng/ml), d, C2IN (100 ng/ml) plus C2IN-C3Stau (100 ng/ml), e, C2IN (100 ng/ml) plus C2IN-C3bot (100 ng/ml). B, intracellular ADP-ribosyltransferase activity of chimeric C3Stau and C3bot were tested by differential ADP-ribosylation. The lysates of treated and nontreated NIH3T3 cells were [32P]ADP-ribosylated with C3Stau. The proteins were resolved on SDS-PAGE and analyzed by phosphorimaging.
ADP-ribosyltransferase from S. aureus

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