Exchange of Glutamine-217 to Glutamate of *Clostridium limosum* Exoenzyme C3 Turns the Asparagine-Specific ADP-Ribosyltransferase into an Arginine-Modifying Enzyme†

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ABSTRACT: C3-like ADP-ribosyltransferaseses are produced by *Clostridium* species, *Bacillus cereus*, and various *Staphylococcus aureus* strains. The exoenzymes modify the low-molecular-mass GTPases RhoA, B, and C. In structural studies of C3-like exoenzymes, an ARTT-motif (ADP-ribosylating turn−turn motif) was identified that appears to be involved in substrate specificity and recognition (Han, S., Arvai, A. S., Clancy, S. B., Tainer, J. A. (2001) *J. Mol. Biol.* 305, 95−107). Exchange of Gln217, which is a key residue of the ARTT-motif, to Glu in C3 from *Clostridium limosum* results in inhibition of ADP-ribosyltransferase activity toward RhoA. The mutant protein is still capable of NAD-binding and possesses NAD^+ glycohydrolase activity. Whereas recombinant wild-type C3 modifies Rho proteins specifically at an asparagine residue (Asn41), Gln217Glu-C3 is capable of ADP-ribosylation of poly-arginine but not poly-asparagine. Soybean trypsin inhibitor, a model substrate for many arginine-specific ADP-ribosyltransferases, is modified by the Gln217Glu-C3 transferase. Also in C3 ADP-ribosyltransferases from *Clostridium botulinum* and *B. cereus*, the exchange of the equivalent Gln residue to Glu blocked asparagine modification of RhoA but elicited arginine-specific ADP-ribosylation. Moreover, the Gln217Glu-C3lim transferase was able to ADP-ribosylate recombinant wild-type C3lim at Arg86, resulting in decrease in ADP-ribosyltransferase activity of the wild-type enzyme. The data indicate that the exchange of one amino acid residue in the ARTT-motif turns the asparagine-modifying ADP-ribosyltransferases of the C3 family into arginine-ADP-ribosylating transferases.

Various bacterial toxins and effectors interfere with eukaryotic cell functions by ADP-ribosylation of essential cellular proteins (*1*). A typical example is the family of C3-like ADP-ribosyltransferases (*2*−*4*). It comprises the prototype *Clostridium botulinum* exoenzyme C3bot[1] (*5*−*7*), including the isoforms C3bot1 and C3bot2, the exoenzyme from *Clostridium limosum* (C3lim) (*8*), *Bacillus cereus* C3 transferase (C3cer) (*9*), and various transferases from *Staphylococcus aureus*, including C3stau1, formerly known as EDIN A (*10*), C3stau2 (EDIN B) (*11*), and C3stau3 (EDIN C) (*12*). The members of this enzyme family are basic 23−25 kDa proteins, which are 30−77% identical to each other in their amino acid sequences.

C3-like ADP-ribosyltransferases specifically modify the small GTP-binding proteins RhoA, B, and C by transferring the ADP-ribose moiety of NAD onto the acceptor amino acid asparagine41 (*2*, *13*, *14*). This leads to the biological inactivation of the Rho proteins and to inhibition of signal processes governed by the GTPases (*8*, *15*−*17*). Additionally, C3stau2 from *S. aureus* strain HMI6 modifies RhoE and Rnd3, which are two recently identified GTP-binding proteins (*11*). Like other ADP-ribosyltransferases, C3-like exoenzymes possess NAD glycohydrolase activity of unknown biological significance.

Although C3-like ADP-ribosyltransferases share only limited sequence similarity, key amino acid residues and short peptide stretches are conserved in all of them. Recently, the crystal structures of C3bot1 either unbound or bound to NAD were solved (*18*, *19*). Furthermore, the structure of C3stau was reported (*20*). Together with data derived from biochemical studies and site-directed mutagenesis (*21*, *22*), the crystal structure analysis largely improved our understanding of the mechanism of the ADP-ribosylation reaction of Rho-ADP-ribosylating transferases. Accordingly, the active site of the exoenzymes is formed by an α-helix bent

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[1] Abbreviations: C3bot, C3 ADP-ribosyltransferase from *Clostridium botulinum*; ARTT-motif, ADP-ribosylation toxin turn−turn motif; C., *Clostridium*; C3lim, C3 ADP-ribosyltransferase from *Clostridium limosum*; C3cer, C3 ADP-ribosyltransferase from *Bacillus cereus*; EXE-C3lim, Gln217Glu mutant of C3 ADP-ribosyltransferase from *C. limosum*; EXE-C3cer, Gln183Glu mutant from *B. cereus*; EXE-motif, motif of 3 amino acid residues in the catalytic site of arginine-ADP-ribosylating transferases, consisting of glutamate, any amino acid, and the "catalytic" glutamate; QXE-motif, motif of 3 amino acid residues in the catalytic site of asparagine-ADP-ribosylating transferases, consisting of glutamine, any amino acid, and the "catalytic" glutamate; MTX, mosquitocidal toxin; GST, glutathione *S*-transferase; SBTI, soybean trypsin inhibitor; WT-C3lim, recombinant wild-type C3 ADP-ribosyltransferase from *C. limosum*.

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over a β-strand, surrounded by two β-strands, in which key amino acids are located. Key residues include a C-terminally located glutamate, termed "catalytic glutamate" and a glutamine residue two positions upstream (QXE-motif). Together with a phenylalanine or tyrosine, these residues are part of an "ADP-ribosylation toxin turn−turn (ARTT) motif", which is suggested to be involved in substrate recognition (*18*). In contrast to the C3-like transferases, which are characterized by the so-called QXE-motif, including the catalytic glutamate, many ADP-ribosyltransferases possess an EXE-motif at the equivalent position (*23*, *24*). Notably, all arginine-modifying enzymes carry this EXE-motif, including cholera toxin, the actin-ADP-ribosylating toxins such as *C. botulinum* C2 toxin, *Pseudomonas aeruginosa* exoenzymes S and T, and the mosquitocidal toxin MTX from *Bacillus sphaericus* (*1*). Here, we studied the role of the glutamine residue in the QXE-motif of C3-like exoenzymes on substrate specificity of the transferases and report that the exchange of the QXE-motif into the EXE-motif in C3 transferases blocks ADP-ribosylation of asparagine residues but allows modification of arginine residues by the enzymes.

EXPERIMENTAL PROCEDURES

Material and Chemicals. Oligonucleotides were obtained from MWG (Ebersberg, Germany). The pGEX-2T vector and the glutathione *S*-transferase Gene Fusion system were from Pharmacia Biotech (Uppsala, Sweden); the Quick-Change Kit was from Stratagene (Heidelberg, Germany). [Adenylate-^{32}P]NAD was from NEN (Belgium). All other chemicals were from commercial sources.

Construction of C3lim Mutants. C3lim mutants proteins were constructed by site-directed mutagenesis with the pGEX-2T−C3lim plasmid as a template (*7*) and the respective oligonucleotides using the Quick-Change Kit according to the manufacturer's instructions. From two complementary primers that were needed, only one is indicated: **R61A**, 5′-CAA ATA TAA GCT GGC TAC AAA CAA AG-3′; **R86A**, 5′-GAA TTT TTA AAA ATG CAT GCG G-3′; **R151A** 5′-TAT AAA AGT GTA TAA AGG GGA TGA TGC-3′; **Q217E**, 5′-CT TTA AAG GTG AAC TTG AAG TGT TGC TTC-3′. Mutated plasmids were transformed in *Escherichia coli* TG1 cells, and all mutations were confirmed by DNA sequencing. Proteins were expressed as GST-fusion proteins or as thrombin-cleaved proteins as described below.

Expression of the Recombinant C3lim Protein. For expression of the recombinant wild-type and mutant C3 exoenzymes, proteins were expressed as glutathione *S*-transferase fusion proteins in *E. coli* as described previously (*7*). For elution as GST-fusion proteins, a buffer containing 50 mM Tris (pH 8.0), 100 mM NaCl, and 10 mM glutathione (reduced form) was used. For cleaving the GST-carrier, the recombinant GST-fusion proteins were incubated with thrombin (3.25 NIH units/mL) in a buffer, containing 150 mM NaCl, 2 mM $CaCl_2$, and 5 mM TEA (pH 7.4) for 45 min at 37 °C. Thrombin was removed by incubation with benzamidine beads (Amersham Pharmacia Biotech), which were precipitated by centrifugation. To control purification and cleavage, GST- and thrombin-cleaved C3lim proteins were subjected to SDS−polyacrylamide gel electrophoresis (SDS−PAGE).

ADP-Ribosylation of RhoA, Soybean Trypsin Inhibitor, and WT-C3lim. Two micrograms of recombinant RhoA, *Soybean Trypsin Inhibitor* (SBTI), or WT-C3lim was incubated with GST-Q217E-C3lim protein or GST-Q183E-C3cer (1 μM) in a buffer, containing 50 mM HEPES (pH 7.4), 2 mM $MgCl_2$, and 50 μM of [adenylate-^{32}P]NAD at various temperatures. Radiolabeled proteins were analyzed by SDS−PAGE, according to the method of Laemmli (*25*). Gels were stained with Coomassie Brilliant Blue R250, distained, and dried. Radiolabeled proteins were analyzed by phophorimaging. The amount of incorporated [^{32}P]ADP-ribose was calculated from Phosphorimager data.

For determination of the specific ADP-ribosyltransferase activities of mutant C3-like exoenzymes, recombinant RhoA was ADP-ribosylated as described above. Specific activities for RhoA were calculated from the linear phase of the reaction with WT-C3lim (10 nM), Q217E-C3lim, and E219Q-C3lim (1 μM) (all as GST-fusion proteins) or WT-C3lim (10 nM final) and R86A-C3lim (200 nM final) using Phosphorimager data. Given are relative enzyme activities. Recombinant wild-type activity was set as 100% and SE is given from three independent experiments.

ADP-Ribosylation of HeLa Cell Lysate. Protein amount of HeLa cell lysate was calculated according to Bradford. Thirty micrograms of total protein was incubated either with the GST-fusion proteins of WT-C3lim (10 nM) or Q217E-C3lim protein (1 μM) for 15 min at 37 °C in a buffer, containing 50 mM HEPES (pH 7.4), 2 mM $MgCl_2$, and 100 μM [adenylate-^{32}P]NAD. Radiolabeled proteins were subjected to SDS−PAGE and, subsequently, analyzed by phosphorimaging. The amount of incorporated [^{32}P]ADP-ribose was calculated from Phosphorimager data.

ADP-Ribosylation of Recombinant Wild-Type C3 Proteins by Q217E-C3lim. One microgram of WT-protein of the indicated C3 transferase was incubated with GST-Q217E-C3lim (1 μM) in a buffer, containing 50 mM HEPES (pH 7.4), 2 mM $MgCl_2$, and 50 μM of [adenylate-^{32}P]NAD, for 30 min at 37 °C. Radiolabeled proteins were subjected to SDS−PAGE and, subsequently, analyzed by phosphorimaging. The amount of incorporated [^{32}P]ADP-ribose was calculated from Phosphorimager data.

Analysis of the Stability of the ADP-Ribose Linkage Formed by Q217E-C3lim. Two micrograms of WT C3lim was incubated with GST-Q217E-C3lim protein (1 μM) in a buffer, containing 50 mM HEPES (pH 7.4), 2 mM $MgCl_2$, and 50 μM [adenylate-^{32}P] NAD, for 10, 20, 40, and 90 min, respectively. For controls, 1 μg RhoA or $\beta\gamma$-actin was incubated with either WT C3lim (20 nM) or recombinant wild-type C2I protein (20 nM) under the same conditions for 15 min at 37 °C. Radiolabeled proteins were subjected to SDS−PAGE and, subsequently, analyzed by phosphorimaging. A second set of samples from a simultaneously performed assay was incubated with hydroxylamine (0.5 M, pH 7.5) for another 2 h. Radiolabeled proteins were analyzed as described above.

ADP-Ribosylation of Mutant C3lim Proteins by Q217E-C3lim. One microgram of C3lim mutant proteins (R61A, R86A, R151A) was incubated with GST-Q217E-C3lim (1 μM) in a buffer, containing 50 mM HEPES (pH 7.4), 2 mM $MgCl_2$, and 50 μM of [adenylate-^{32}P]NAD, for 15 min at 37 °C. Radiolabeled proteins were subjected to SDS−PAGE and, subsequently, analyzed by phosphorimaging. The amount of incorporated [^{32}P] ADP-ribose was calculated from Phosphorimager data.

Determination of the Transferase Activity of WT-C3lim ADP-Ribosylated by GST-Q217E-C3lim. Recombinant wild-type C3lim (1 μM) was incubated with and without GST-EXE-C3lim fusion protein (3 μM) in a buffer, containing 50 mM HEPES (pH 7.4), 2 mM $MgCl_2$, and 200 μM NAD, for 2 h at 30 °C. In a subsequent ADP-ribosylation reaction, 2 μg of recombinant RhoA was incubated with previously ADP-ribosylated or buffer-incubated WT C3lim (10 nM) in the same buffer as described above in the presence of 100 μM [adenylate-^{32}P]NAD for 1, 2, 4, and 8 min, respectively. Radiolabeled proteins were analyzed as described above.

NAD Glycohydrolase Reaction. For detection of NAD glycohydrolase activity, 200 μM [adenylate-^{32}P]NAD were incubated with various concentrations of either WT-C3lim or mutant C3lim proteins in a buffer, containing 50 mM HEPES (pH 7.3) and 2 mM $MgCl_2$, at 37 °C for up to 2 h. Five-microliters aliquots of the reaction mixture were separated by TLC on Silica Gel 60_{F254} (Merck, Germany) with 66% 2-propanol and 0.33% ammonium sulfate, followed by phosphorimaging. The amount of cleaved [^{32}P]ADP-ribose was calculated from Phosphorimager data.

Detection of ADP-Ribosylation by the Filter Assay. One hundred micrograms of poly-L-arginine or poly-L-asparagine was incubated with either WT-C3lim protein or mutant Q217E-C3lim in form of the respective GST-fusion protein (100 nM) in a buffer, containing 50 mM HEPES (pH 7.0), 3 mM EDTA, 2 mM $MgCl_2$, and 10 mM dithiothreitol, in the presence of 10 μCi [adenylate-^{32}P]NAD in a total volume of 100 μL for 30 min at 30 °C. Afterwards, the samples were precipitated on Whatman-disks soaked with TCA 10%. These were washed four times with 1 mL of trichloroacetic acid (5%). Incorporated radioactivity was measured by scintillation counting.

For time courses, 100 μg of poly-L-arginine was incubated with 500 nM WT-C3lim protein or 100 nM Q217E-C3lim, without and with ADP-ribose (100 μM final) under conditions described above for 5, 10, 20, and 40 min, respectively, at 30 °C.

RESULTS

Consequences of Exchange of Gln217 of C3lim on ADP-Ribosylation of RhoA and NAD Glycohydrolase Activity. In *C. limosum* C3 transferase (C3lim), Gln217 is located in the "ARTT-motif" of the active site, two positions upstream of the catalytic glutamate (Glu219). Exchange of Gln217 to Glu (EXE-C3lim) blocked the ADP-ribosylation activity of C3 to modify recombinant RhoA (Figure 1). Decrease in Rho-modifying activity was comparable with the extent of inhibition caused by exchange of the catalytic glutamate in position 219 to glutamine, resulting in the inactive QXQ-C3 enzyme (Table 1). In contrast, the NAD glycohydrolase activity of the EXE mutant was much less affected than the ADP-ribosyltransferase activity. Whereas the NAD glycohydrolase activity of the QXQ-C3 mutant was inhibited by >99%, the EXE mutant exhibited about 33% activity as compared to the wild-type enzyme (Table 1).

EXE-C3 Enzymes ADP-Ribosylate Arginine Residues. Because the EXE-motif is typical for arginine-modifying ADP-ribosyltransferases, we studied the ADP-ribosylation of the mutant C3lim with poly-L-arginine in comparison to poly-L-asparagine. As shown in Figure 2A, EXE-C3lim modified poly-L-arginine to a much larger extent than poly-L-asparagine. On the other hand, the WT-C3lim was not able to modify poly-L-arginine over background level. Also poly-L-asparagine was only slightly modified by WT-C3lim, indicating the high specificity of the enzyme for Rho GTPases. To exclude that labeling of poly-L-arginine was caused unspecifically and secondary to cleavage of NAD possibly caused by the NAD glycohydrolase activity of the enzyme, we performed the reaction in the presence of a high amount of ADP-ribose (100 μM) (Figure 2B). Although the radioactive labeling of poly-L-arginine was slightly reduced with ADP-ribose, a significant labeling was detected, indicating that poly-L-arginine was modified by ADP-ribosylation

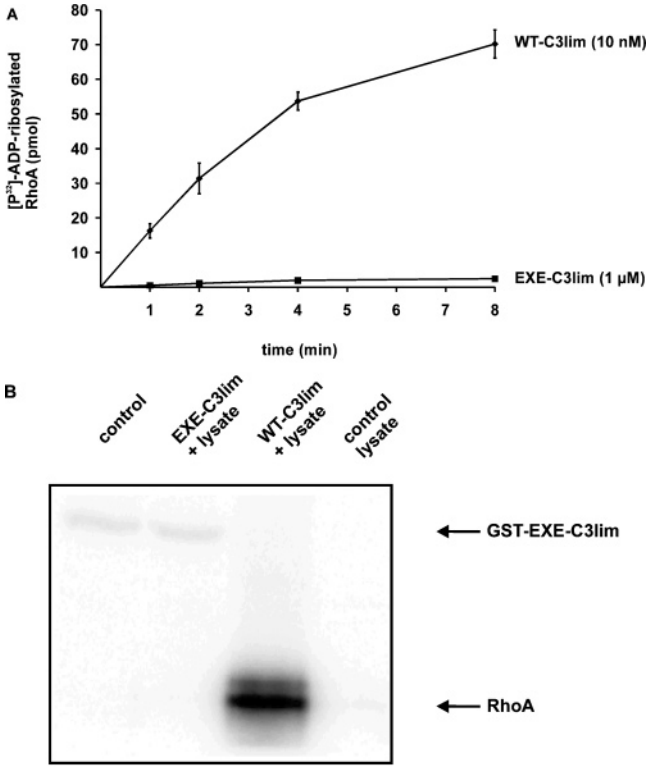

FIGURE 1: ADP-ribosylation of RhoA by wild-type C3lim and by EXE-C3lim. (A) Two micrograms of recombinant RhoA was incubated with the indicated enzymes (GST-fusion proteins) in the presence of [adenylate-^{32}P]NAD for up to 8 min. Radiolabeled proteins were subjected to SDS−PAGE, and the amount of modified RhoA was quantified by phosphorimaging. (B) HeLa cell lysate (30 μg of protein) was incubated with 10 nM wild-type GST-C3lim (WT-C3lim) or 1 μM GST-EXE-C3lim in the presence of [^{32}P]-NAD for 15 min. Radiolabeled proteins were subjected to SDS−PAGE, and the autoradiography is shown.

Table 1: ADP-Ribosyltransferase and NAD Glycohydrolase Activities of Recombinant Wild-Type (WT) C3lim and C3lim Mutants E219Q and Q217E[a]

	specific activity [relative activity in %]	
toxin	transferase	glycohydrolase
WT	100	100
E219Q	<0.01	0.3
Q217E	<0.01	33

[a] The specific ADP-ribosyltransferase- and NAD glycohydrolase activities of the respective GST-fusion proteins were measured as described under Experimental Procedures. For calculation of the relative enzyme activities, wild-type activity was set as 100% and SE is given from three independent experiments. Specific activity of WT-C3lim for RhoA was 80 ± 5.4 ((mol/mol)/min) for transferase activity and 1.1 ± 0.02 ((mol/mol)/min) for NAD glycohydrolase activity.

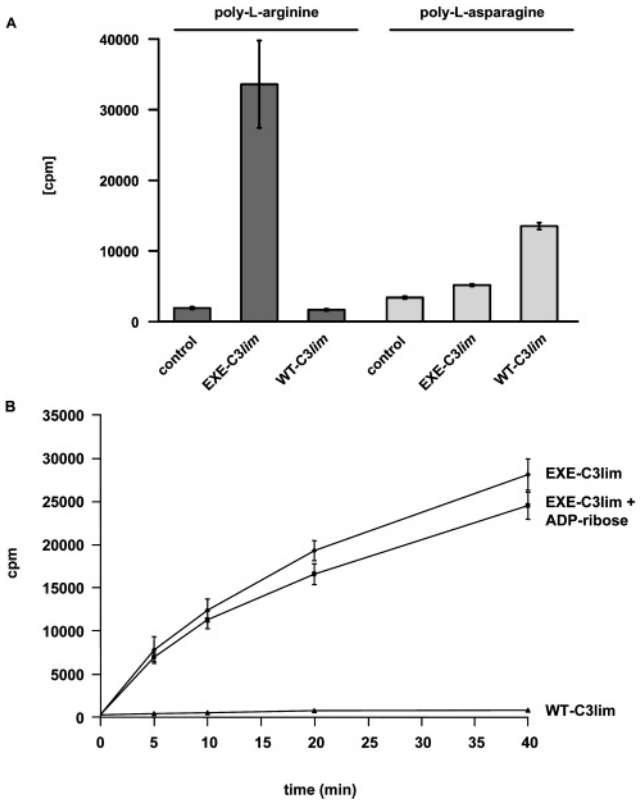

FIGURE 2: ADP-ribosylation of poly-L-arginine or poly-L-asparagine by wild-type C3lim and EXE-C3lim. (A) Poly-L-arginine or poly-L-asparagine (final 1 mg/mL) was incubated with recombinant wild-type GST-C3lim (WT-C3lim) or GST-EXE-C3lim proteins (100 nM final) in the presence of [adenylate-^{32}P]NAD for 30 min at 30 °C. After that time, the samples were spotted onto trichloroacetic acid-treated 10% Whatman paper disks. These were washed four times with 1 mL of trichloroacetic acid 5%. Incorporated radioactivity was measured by scintillation counting. (B) Time course of the ADP-ribosylation of poly-L-arginine (1 mg/mL) by GST-EXE C3lim (100 nM) or recombinant wild-type GST-C3lim (WT-C3lim, final 500 nM) in the presence and absence of 100 μM ADP-ribose.

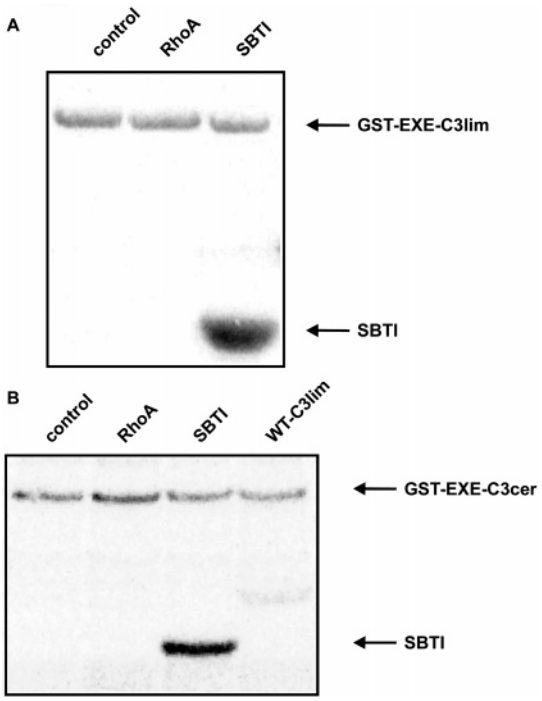

FIGURE 3: ADP-ribosylation of soybean trypsin inhibitor by EXE-C3lim and EXE-C3cer. (A) ADP-ribosylation of soybean trypsin inhibitor protein (SBTI) and RhoA by EXE-C3lim. SBTI or recombinant RhoA (2 μg of protein each) was incubated with GST-EXE-C3lim (100 nM final) in the presence of [adenylate-^{32}P]NAD for 30 min at 37 °C. (B) ADP-ribosylation of soybean trypsin inhibitor protein (SBTI), RhoA, and recombinant wild-type C3lim (WT-C3lim) by Q183E-C3cer (EXE-C3cer). Two micrograms of SBTI protein, recombinant RhoA, or wild-type C3lim (WT-C3lim) was incubated with GST-EXE-C3cer for 30 min at 37 °C.

and not labeled as the consequence of the NAD glycohydrolase activity of the enzyme.

Many arginine-modifying ADP-ribosyltransferases, including the active fragment of *B. sphaericus* transferase MTX (*26*), are capable of modifying soybean trypsin inhibitor (SBTI). Also EXE-C3lim modified this protein (Figure 3A). In addition, we studied the ability of other C3-like transferases to modify SBTI. We observed that also EXE-C3cer (Figure 3B) and EXE-C3bot (not shown) labeled SBTI in the presence of [^{32}P]NAD. C3lim modifies RhoA at Asn41 (*13*). Because the EXE-C3 mutant ADP-ribosylated arginine residues, we wondered whether change of asparagine to arginine at position 41 of RhoA allows modification by EXE-C3. This was not the case, indicating that additional structural requirements define modification at asparagine 41 in RhoA (data not shown).

EXE-C3lim ADP-Ribosylates Recombinant Wild-Type C3lim. In the course of the above-described studies, we observed that the EXE-C3lim was able to modify itself to some extent (see Figure 3). To clarify whether this was the result of an intra- or intermolecular ADP-ribosylation, we studied this type of ADP-ribosylation by EXE-C3lim in the presence of high concentrations of wild-type C3lim. We observed that the wild-type enzyme was effectively labeled by EXE-C3lim. In contrast, other C3 exoenzymes such as C3bot, C3stau2, and C3cer were not or much less modified by EXE-C3lim (Figure 4A). Furthermore, the EXE-mutants of C3bot (not shown) and C3cer (Figure 3B) were not able to modify wild-type C3lim to the same extent as EXE-C3lim, indicating that specific structural requirements must be fulfilled for the modification.

Next, we tried to get more insight into the preferential modification of wild-type C3lim by EXE-C3lim. At first, we confirmed that arginine residues were modified by the mutant transferases. For this purpose, wild-type C3lim was [^{32}P]ADP-ribosylated by EXE-C3lim for 90 min. Thereafter, the modified protein was treated with neutral hydroxylamine, which is known to split the ADP-ribose-arginine bonds but not the bonds that formed between ADP-ribose and asparagine (*27*−*29*). As shown in Figure 4B, hydroxylamine treatment largely reduced the labeling of wild-type C3lim, indicating modification of an arginine residue. As controls, actin ADP-ribosylated at arginine by C2 toxin was also cleaved, whereas the modification of RhoA at asparagine was only minimally reduced by hydroxylamine. Because wild-type C3lim was modified by EXE-C3lim at an arginine residue, we examined, by using the available 3D structures of C3, which arginine residues of C3lim are located on the surface of the molecule. At least three arginine residues Arg61, Arg86 ,and Arg151 were identified to be located at the protein surface. Subsequently, we changed these residues with alanine. Then, we studied the ability of the mutant proteins to serve as substrates for EXE-C3lim. As shown in

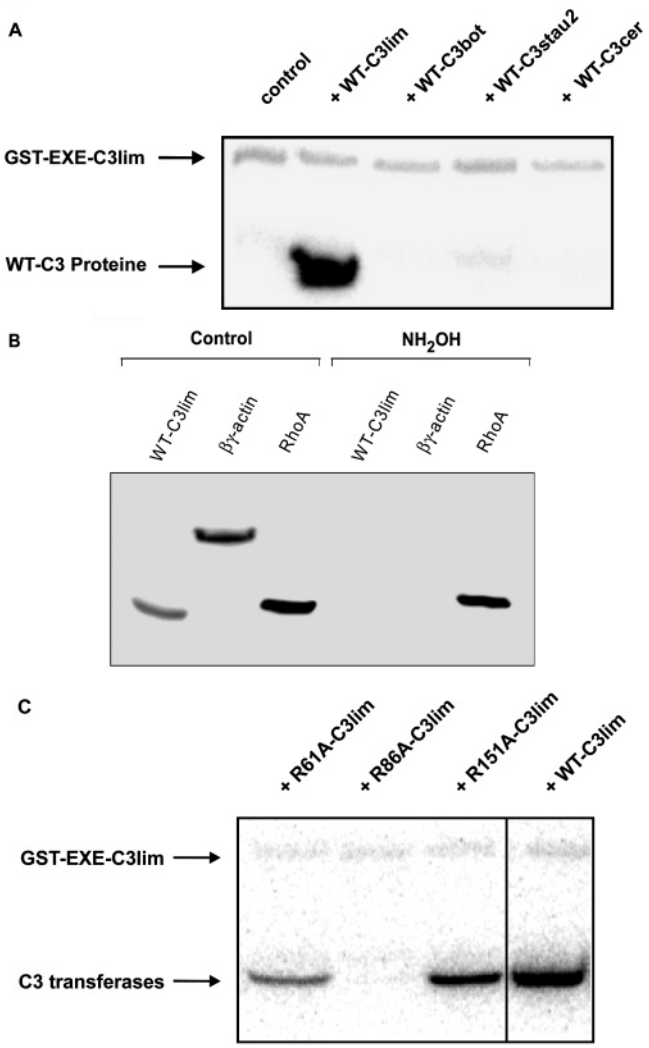

FIGURE 4: ADP-ribosylation of WT-C3lim by EXE-C3lim. (A) ADP-ribosylation of wild-type C3 transferases by EXE-C3lim. Recombinant wild-type C3 transferases (WT-C3lim, WT-C3bot, WT-C3stau2, and WT-C3cer, 1 μg each) were incubated with GST-EXE-C3lim, 1 μM final in the presence of [adenylate-^{32}P]NAD for 30 min at 37 °C. Radiolabeled proteins were subjected to SDS−PAGE, and the amount of modified RhoA was quantified by phosphorimaging. (B) Analysis of the stability of the ADP-ribose-linkage formed by EXE-C3lim. Recombinant wild-type C3lim (WT-C3lim), $\beta\gamma$-actin, and RhoA (each 1 μg of protein) were ADP-ribosylated by GST-EXE-C3lim (1 μM), C2I (20 nM), and recombinant wild-type C3lim (20 nM), respectively, in the presence of [adenylate-^{32}P]NAD (control). NH_2OH (0.5 M final) was added to aliquots, and samples were incubated for another 2 h at 37 °C. Radiolabeled proteins were subjected to SDS−PAGE, and the amount of modified RhoA was quantified by phosphorimaging. (C) ADP-ribosylation of mutant C3lim proteins by EXE-C3lim. C3lim mutant proteins (R61A-C3lim, R86A-C3lim, and R151A-C3lim, each 1 μg, indicated as C3 transferases) or recombinant wild-type C3lim (WT-C3lim) was incubated with GST-EXE-C3lim in the presence of [adenylate-^{32}P]NAD for 30 min. Radiolabeled proteins were subjected to SDS−PAGE, and the amount of modified RhoA was quantified by phosphorimaging.

Figure 4C, change of Arg86 to alanine resulted in inhibition of ADP-ribosylation of wild-type C3lim by EXE-C3lim, indicating that the ADP-ribosylation occurs at this arginine residue. Alignment of C3lim and C3bot1, which share 67% identity in their amino acid sequence, showed that the arginine, which is modified by EXE-C3lim, is not present in C3bot.

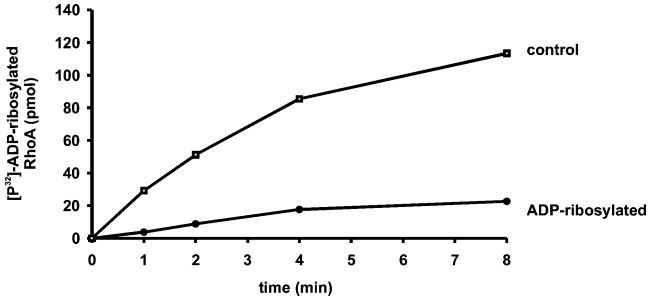

FIGURE 5: Time-dependent ADP-ribosylation of RhoA by ADP-ribosylated C3lim. WT-C3lim (1 μM final) was ADP-ribosylated with and without GST-EXE C3lim (3 μM) in the presence of NAD (200 μM) for 2 h at 30 °C. The remaining RhoA-specific ADP-ribosyltransferase activity was quantified in a time-dependent manner by measuring the ADP-ribosylation of RhoA (2 μM) in the presence of 100 μM [^{32}P]NAD. For control, RhoA was ADP-ribosylated with WT-C3lim, which was pretreated without GST-EXE-C3lim. Radiolabeled proteins were subjected to SDS−PAGE, and the amount of modified RhoA was quantified by phosphorimaging.

Table 2: ADP-Ribosyltransferase- and NAD Glycohydrolase Activities of Recombinant Wild-Type (WT) C3lim and C3lim Mutant R86A[a]

toxin	ADP-ribosyltransferase activity [relative activity in %] RhoA	NAD glycohydrolase activity [relative activity in %]
C3lim WT	100	100
C3lim R86A	8.1 ± 1.9	51 ± 3.8

[a] The specific ADP-ribosyltransferase- and NAD glycohydrolase activities were measured as described under Experimental Procedures. For calculation of the relative enzyme activities, wild-type activity was set as 100% and SE is given from three independent experiments. Specific activity of WT-C3lim for RhoA was 80 ± 5.4 ((mol/mol)/min) for transferase activity and 1.1 ± 0.02 ((mol/mol)/min) for NAD glycohydrolase activity.

Functional Consequences of ADP-Ribosylation of Arg86 of Recombinant Wild-Type C3lim. Next we studied, whether the ADP-ribosylation of WT-C3lim at Arg86 has any functional consequences for its enzyme activity. To this purpose, WT-C3lim was treated with EXE-C3lim, which was bound to Sepharose beads. After incubation for 120 min, WT-C3 was separated from EXE-C3 by centrifugation and the modified WT-C3 was used in an ADP-ribosylation assay with RhoA. As shown in Figure 5, the ADP-ribosylation of WT-C3 at Arg86 reduced the transferase activity by about 50%. In contrast, the NAD glycohydrolase activity of ADP-ribosylated WT-C3 was not decreased (not shown), indicating that modification of WT-C3 at Arg86 is important for the enzyme−protein substrate interaction. This was confirmed by change of Arg86 of C3lim to alanine, resulting in decrease of transferase activity by 90%, whereas NAD glycohydrolase activity was reduced only by about 20% (Table 2).

DISCUSSION

Up to the present, seven different C3 transferases with sequence identities between 30 and 77% have been described. All these transferases modify Rho-GTPases at asparagine41. With the exception of C3stau2 transferase, which additionally modifies RhoE, all the other transferases ADP-ribosylate RhoA, B, and C, whereas other GTPases (so far studied) are very poor (Rac1) or not all substrates (Cdc42) (*4*). At least three crystal structures are available for C3bot from *C.*

		turn1 turn2	
C3lim	210	PISTFKG**Q**L**E**VLL	222
C3bot1	205	PISYFPG**Q**L**E**VLL	217
C3cer	176	DLTAYPG**Q**Y**E**LLL	188
C3stau2	196	ELTAYPG**Q**Q**E**VLL	208
C2 toxin	380	GFSGFQD**E**Q**E**ILL	393
VIP2	419	AIGGFAS**E**K**E**ILL	431
PT	120	AGALATY**Q**S**E**YLA	132
MTX	188	NHNPFPN**E**D**E**ITF	200
Pierisin1	156	DASPWPN**Q**M**E**VAF	169
ExoS	372	GISNYKN**E**K**E**ILY	384
CT	103	AISPHPD**E**Q**E**VSA	115
rART2.2	180	EFSFRPD**Q**E**E**VLI	192

FIGURE 6: Alignment of the active site of various ADP-ribosyltransferases. Residues of the ADP-ribosylating turn−turn motif (ARTT) are shown. The EXE- and QXE-motifs, consisting of the conserved catalytic glutamate and the proceeding glutamine/glutamate residues of turn 2 of the ARTT-motif, which define the acceptor amino acid for ADP-ribosylation, are indicated (bold). Numbering of the C3-like transferases are with signal sequences except for C3cer and C3stau2. (C3lim, *C. limosum* C3 exoenzyme; C3bot, *C. botulinum* C3 exoenzyme; C3cer, *B. cereus* C3 exoenzyme; C3stau2, *S. aureus* C3 exoenzyme; C2, *C. botulinum* C2 toxin; VIP2, *B. cereus* vegetative insecticidal protein; PT, pertussis toxin; MTX, *B. sphaericus* mosquitocidal toxin; Pierisin1, toxin from *Pieris rapae*; ExoS, *P. aeruginosa* exoenzyme S; CT, cholera toxin; rART2.2, rat ecto-ADP-ribosyltransferase ART2.2.

botulinum and C3stau2 from *S. aureus*, respectively (*18*−*20*). These structure analyses and recent mutational studies allow major insights into the structure of the active site of this enzyme family and reveal a folding of the catalytic domain of C3 enzymes very similar to that of other ADP-ribosyltransferases. All of these enzymes possess a catalytic glutamate, which is essential for the ADP-ribosylation and NAD glycohydrolase activities of the enzymes (*23*, *24*) (Figure 6). All arginine-modifying ADP-ribosyltransferases possess another glutamate residue (EXE-motif) two amino acid residues upstream of the catalytic glutamate. Change of this residue blocks the transferase activity but not the NAD glycohydrolase activity. In C3 transferases, which specifically attach ADP-ribose to asparagine, a glutamine residue (QXE-motif) is at this position. Pertussis toxin, which modifies cysteine residues by ADP-ribosylation (*30*, *31*), also has a QXE-motif. Glutamines are also found at the equivalent position in pierisin, an ADP-ribosyltransferase from cabbage butterfly (*32*), which was shown to modify DNA at guanosine residues (*33*) and in the ecto-ADP-ribosyltansferase-like protein rART2B from rats (*34*), which is a strong NAD glycohydrolase but not a transferase. The rART2B ecto-ADP-ribosyltransferase is of particular interest, because change of Gln of the QXE-motif to Glu increased significantly the transferase activity (*35*). We showed previously that exchange of this glutamine residue in C3 transferases blocks ADP-ribosylation of Rho, whereas the NAD glycohydrolase activity is hardly affected (*22*, *36*). Here, we confirmed that exchange of this glutamine residue blocks the ADP-ribosylation of RhoA. Surprisingly, we observed that the enzyme still possessed a transferase activity toward arginine residues. Thus, exchange of one amino acid residue turns the asparagine-modifying enzyme into an arginine-modifying transferase. This was first studied with poly-L-arginine in comparison to poly-L-asparagine. Modification of poly-L-arginine by EXE-C3 was not blocked by the addition of ADP-ribose, indicating that an ADP-ribosylation reaction and not a secondary reaction with ADP-ribose formed by the NAD glycohydrolase activity of C3, was responsible for the modification. Importantly, WT-C3lim was not able to modify poly-L-arginine, indicating that ADP-ribosylation by EXE-C3lim is not a residual activity also found in the wild-type enzyme. Similar to many arginine-modifying ADP-ribosyltransferases, which are characterized by a broad in vitro substrate specificity like *P. aeruginosa* exoenzyme S (*37*, *38*) and *B. sphaericus* ADP-ribosyltransferase MTX (*26*), we observed that soybean trypsin inhibitor (SBTI) was a protein substrate of EXE-C3lim. Moreover, other asparagine-specific C3 transferases such as C3bot from *C. botulinum* and C3cer from *B. cereus* were capable of modifying arginine residues after exchange of the equivalent glutamine residues.

Whereas the catalytic reaction, which causes splitting of NAD by ADP-ribosyltransferases in nicotinamide and ADP-ribose is well-understood, the structural determinants underlying the substrate specificity, including selectivity for certain acceptor amino acid residues and high preferences for specific protein substrates of the transferases, are still enigmatic. Recently, Tainer and co-workers suggested that a region termed ADP-ribosylating toxin turn−turn (ARTT) motif is involved in substrate recognition (*18*). This motif covers residues Ser207 through Glu214 in C3bot. Turn 1 of the motif contains a hydrophobic phenylalanine residue, which was suggested to interact with a hydrophobic pocket of the substrate RhoA located in the vicinity of the acceptor amino acid asparagine 41. Turn 2 is mainly built by the QXE-motif with the solvent-exposed Gln212 and the catalytic Glu214 at the basis. It was suggested that Gln212 of C3bot interacts with Asn41 of RhoA by a pair of hydrogen bonds between carbonyl and amide groups of the acceptor amino acid Asn41. Moreover the N−Q interaction between Rho and C3 is proposed to be necessary for a correct postitioning of the amide N of the acceptor amino acid for the nucleophilic attack on C1 of the ribose. A model for the role of the respective residues Gln217 and Glu219 of C3lim is shown in Figure 7A. Exchange of the equivalent glutamine residue to glutamate blocks the asparagine specific ADP-ribosylation of RhoA by EXE-C3lim and EXE-C3cer. With respect to the proposed function of the glutamine residue of the QXE-motif, this inhibition is feasible, because the introduced glutamate residue can only form one hydrogen bond with Asn41 of RhoA (Figure 7B). This destabilizes the orientation and the distance of Asn41 of RhoA to the C1 ribose of NAD and renders RhoA to a nonsubstrate for EXE-C3 enzymes. However, arginine residues from either poly-L-arginine or SBTI can be recognized by EXE-C3 via an R−E interaction (Figure 7C). Glu217 of EXE-C3lim may act in deprotonation of an arginine acceptor amino acid to increase the nucleophilicity for attack on C1.

A preferential interaction between N−Q residues and R−E residues was also recently described from a biophysical analysis of protein−protein interactions (*39*). Furthermore, an R−E interaction seems to be less specific than the Q−N interaction, because it is independent of H-bonds and not restricted to a fixed angle. This may explain the arginine modification of model substances (poly-L-arginine and SBTI) by EXE-C3 enzymes. It is noteworthy that the actin-ADP-

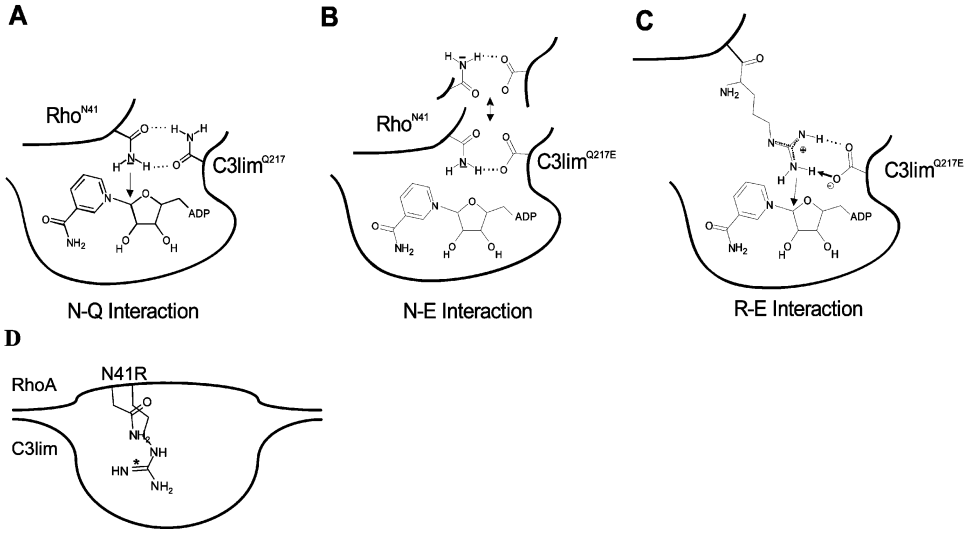

FIGURE 7: Model for the interaction of asparagine or arginine with QXE- or EXE-C3lim. A scheme of the catalytic core of C3, containing the nicotinamide-ribose part of NAD is shown. (A) C3lim^{Q217}, which is located in the ARTT-loop, recognizes Asn41 od RhoA via two hydrogen bonds. Because of the Q−N interaction, Asn41 of RhoA is in a favorable position for nucleophilic attack on C1 of the ribose (asterisks). (B) Introduction of C3lim-Q217E exchanges the QXE-motif to a EXE-motif, which is conserved in arginine-modifying toxins. EXE-C3lim can only interact via one H-bond with Asn41 of RhoA. As a consequence, Asn41 of RhoA is destabilized and the asparagine-specific ADP-ribsoylation of RhoA by EXE-C3lim is blocked. (C) 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. (D) Model for inhibition of ADP-ribosylation of Asn41Arg−RhoA by EXE-C3. The recognition of RhoA by C3lim is schematically shown. Asn41 of RhoA is suggested to be located in the catalytic core of C3. After exchange of Asn41 to arginine, RhoA still is recognized by C3lim. The increased length leads to a sterical disordering of the arginine in the catalytic core of C3lim and, therefore, excludes a favorable localization for ADP-ribosylation in the vicinity of the C1 of the ribose of NAD (indicated by an asterisk).

ribosylating toxins VIP2 and iota toxin, which modify actin at arginine 177, are also suggested to possess equivalent ARTT-loops, containing the EXE-motifs in turn 2 (*18*, *40*, *41*). Therefore, we were prompted to change Asn in RhoA to Arg to test whether EXE-C3 was able to modify Asn41Arg−RhoA. However, this was not the case. The effective transfer of the ADP-ribose moiety was blocked probably due to additional methylene group of the introduced arginine (Figure 7D). Therefore, this RhoA mutation cannot be used to analyze a specific modification by EXE-C3. Thus, the hypothesis that the hydrophobic residue of turn 1 of the ARTT-loop defines the affinity for the protein substrate (RhoA in the case of C3 exoenzymes) and turn 2 (with the glutamine in the case of C3 exoenzymes and glutamate in arginine-modifying enzymes) recognizes the acceptor amino acid is not sufficient to explain the substrate specificity of C3 exoenzymes. Additional residues are obviously also involved in recognition of the protein substrate by C3.

We observed that EXE-C3lim but not (or much less) QXE-C3lim was labeled in the presence of radioactive NAD. At first, we suggested an auto-ADP-ribosylation, which is described for several ADP-ribosylating enzymes, including *P. aeruginosa* exoenzyme S (*42*) or the rat ecto-ADP-ribosyltransferase 2.2 (*43*−*46*). However, the WT-C3lim, containing the QXE-motif was a good substrate for ADP-ribosylation by EXE-C3lim, excluding an intramolecular ADP-ribosylation. The modification of WT-C3lim by EXE-C3lim was specific for C3lim but was not found with C3bot or C3cer. We assumed that an arginine residue at the surface of C3lim was modified and studied possible candidate residues. Because exchange of Arg86 with alanine blocked the ADP-ribosylation, we believe that this residue of WT-C3lim is modified by EXE-C3lim. This finding predicts that C3lim possesses a tendency to interact with each other. Arg86 of C3lim, which was modified by EXE-C3lim, is located at the end of helix α2 and at the begining of helix α3 of WT-C3lim. This residue is near the mouth of the catalytic cleft, which harbors the binding sites for NAD and is in the vicinity of the ARTT loop. Because the modification at Arg86 was complete and it was near the catalytic site, we studied the functional consequences of ADP-ribosylation of Arg86 of WT-C3lim in ADP-ribosylation and NADase assays. ADP-ribosylation at Arg86 largely decreased the ability of WT-C3lim to modify RhoA. In contrast, the NAD glycohydrolase activity was poorly affected. This finding suggests that Arg86 and most likely helix α3 is involved in protein substrate recognition of C3lim. This was also suggested recently from the crystal structure of C3stau2 (*20*). In line with this hypothesis, we observed that exchange of Arg86 in C3lim to alanine largely reduced the ability to modify RhoA. Thus, it is suggested that the surface of C3lim in this region near the mouth of the catalytic cleft of the transferase is particularly suitable for interaction with potential substrates, allowing the intermolecular ADP-ribosylation by the arginine-modifying EXE-C3lim.

Taken together, here we show that an asparagine-ADP-ribosylating enzyme can be changed into an arginine-modifying transferase by exchanging one single residue (Gln to Glu) located in the catalytic site. However, considering that some arginine-modifying ADP-ribosyltransferases are extremely selective with respect to their substrates (e.g., C2 toxin modifies $\beta\gamma$-actin but not α-actin isoforms (*47*)), whereas other enzymes have broad in vitro substrate specificities such as *B. sphaericus* MTX-toxin (*26*, *48*) or *P. aeruginosa* exoenzyme S (*49*, *50*), the complex structural determinants of these specificities have still to be elucidated.

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