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ADP-ribosylation by *Clostridium botulinum* C3 exoenzyme increases steady-state GTPase activities of recombinant rhoA and rhoB proteins

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ADP-ribosylation of recombinant rhoA and rhoB proteins by *Clostridium botulinum* C3 exoenzyme increased steady-state GTP hydrolysis by 50 to 80%. ADP-ribosylation and increase in GTP hydrolysis occurred at similar concentrations of C3, depended on the presence of NAD and were prevented by anti-C3 antibody or heat inactivation of C3. In contrast, GTP hydrolysis by Ile-41 rhoA or Ha-ras, which are no substrates for the transferase, were not affected by C3. ADP-ribosylation facilitated the [^{3}H]GDP release and subsequently, the binding of [^{3}H]GTP to rhoA. The data indicate that the increase in the steady-state GTPase activity by ADP-ribosylation is caused by increasing the rate of GDP release which is suggested to be the rate limiting step of the GTPase cycle of the small GTP-binding proteins.

ADP-ribosylation; *Clostridium botulinum* C3 exoenzyme; GTPase; rho Protein; GTP-binding

1. INTRODUCTION

The small GTP-binding proteins rhoA,B,C belong to the superfamily of ras-related proteins [1,2]. These proteins function by utilizing a guanine nucleotide-binding and hydrolyzing cycle [3–5]. The GTP-binding proteins are active in the GTP-bound form and inactive after hydrolysis of GTP to GDP catalyzed by an associated GTPase activity. Recent studies have shown that the activity state of rho proteins is under the control of regulatory proteins which can either increase the GTPase activity (GTPase stimulating protein, GAP) [6,7], facilitate the guanine nucleotide exchange (guanine nucleotide dissociation stimulator, GDS) [8,9] or inhibit the nucleotide release (guanine nucleotide dissociation inhibitor, GDI) [10,11]. So far, the precise functions of the rho proteins are not clarified. Some evidence has been presented that these proteins are involved in the regulation of the cytoskeleton and in the formation of actin filaments [12–14].

Various GTP-binding proteins are substrates for bacterial ADP-ribosyltransferases. While elongation factor 2 is ADP-ribosylated by diphtheria toxin and *Pseudomonas* exotoxin A, heterotrimeric G-proteins are substrates of cholera- and pertussis toxins [15–18]. ADP-ribosylation of these GTP-binding proteins has been shown to affect their GTP-binding and GTPase activities. The rhoA,B,C proteins are substrates of *Clostridium botulinum* exoenzyme C3 [19–21] which modify the GTP-binding protein in Asparagine-41 [22]. So far no effects of ADP-ribosylation on GTP-binding and/or GTPase activities of rho have been reported [23]. Those studies were mostly performed with rho proteins purified from bovine brain [23,24]. Now, we studied the influence of ADP-ribosylation on GTP binding and GTP hydrolysis of recombinant rho proteins. Here we report that the ADP-ribosylation increases the steady state GTPase activity of recombinant rhoA protein, an effect which was paralleled by an increased release rate of [^{3}H]GDP from the modified GTP-binding protein.

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2. MATERIALS AND METHODS

2.1. *Materials*

Clostridium botulinum ADP-ribosyltransferase C3 was purified as described [19,25]. Recombinant rhoA, rhoB, Val-14 rhoA, Ile-41 rhoA and ras proteins were purified as described [12,26]. The purity of rho proteins and of Ha-ras protein were 30 to 40 and 90%, respectively. The amount of GTP-binding proteins present in the individual preparations used is given in terms of [^{3}H]GTP-binding activity. The anti-C3 antibody used was characterized in [27]. Nucleotides were obtained from Boehringer (Mannheim, Germany). [γ-^{32}P]GTP, [^{3}H]GTP and [^{32}P]NAD were purchased from NEN (Dreieich, Germany).

2.2. *Methods*

GTP hydrolysis assay. GTP hydrolysis was determined in a standard buffer containing 50 mM triethanolamine-HCl (pH 7.5), 1 mM EDTA, 1.1 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin, 0.5 mM phosphatidylcholine, 1 mM dithiothreitol, 50 nM [γ-^{32}P]GTP (about 0.1 μCi/tube) and rho proteins (about 0.1–0.2 μg/tube) in a total volume of 100 μl for the indicated periods of time at 30°C. The reaction was terminated by addition of 700 μl of ice-cold 20 mM sodium phosphate buffer (pH 2), containing charcoal (5%, w/v). After centrifugation (10 min, 13,000 × g) at 4°C, 500 μl of the supernatant were transferred into scintillation vials, 3.5 ml water was added and

the samples were counted for radioactivity. The blank was less than 0.5% of added [^{32}P]GTP and was substracted from counted radioactivity.

In case of the experiment described in Fig. 1A, C3 (0.1 μg), heat-inactivated C3 (0.1 μg, 30 min; 95°C) was preincubated with 10 μl of anti-C3 IgG or heat-inactivated anti-C3 IgG (10 min, 95°C) for 1 h at room temperature. Thereafter, standard buffer, rhoA (0.1 μg) without and with 100 μM NAD were added and incubated for 10 min at 30°C. Thereafter, 50 nM [^{32}P]GTP was added and the reaction was continued for 20 min at 30°C.

For comparison of ADP-ribosylation and GTP hydrolysis (Fig. 1C) the reaction medium contained the standard buffer with either unlabeled or labeled NAD (10 μM; 0.8 μCi [^{32}P]NAD). ADP-ribosylation was performed for 10 min at 30°C. Then 5 μl of anti-C3 IgG were added and incubated for 5 min on ice. Thereafter, [^{32}P]GTP (50 nM; 0.5 μCi) was added for determination of GTP hydrolysis and the reaction continued for 20 min at 30°C. The GTP hydrolysis reaction was terminated as described. The ADP-ribosylation was determined after the addition of 1 ml trichloroacetic acid (30%, w/v). The precipitates were filtered onto nitrocellulose membranes. The membranes were washed with 20 ml trichloroacetic acid (6%, w/v) and counted for radioactivity.

GTP-binding assay. The rho protein preparation (about 0.01–0.02 μg/tube) was incubated for 10 min at 30°C in a buffer containing 50 mM triethanolamine-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 100 μg/ml bovine serum albumin, 0.5 mM phosphatidylcholine, 100 μM NAD and 1.1 mM $MgCl_2$ without and with 0.3 μg C3. Then [^{3}H]GTP was added and the incubation continued at 30°C. At the indicated times two 100 μl aliquots were placed in 1 ml of ice-cold washing buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl and 5 mM $MgCl_2$. The samples were immediately filtered onto nitrocellulose membranes washed four times with 4 ml of washing-buffer and counted for radioactivity.

For determination of the release of bound nucleotide the recombinant rhoA was preloaded with [^{3}H]GTP by incubating 6 μl of the rho solution (0.01–0.02 μg) with 4.2 mM EDTA 1.6 mM $MgCl_2$ and

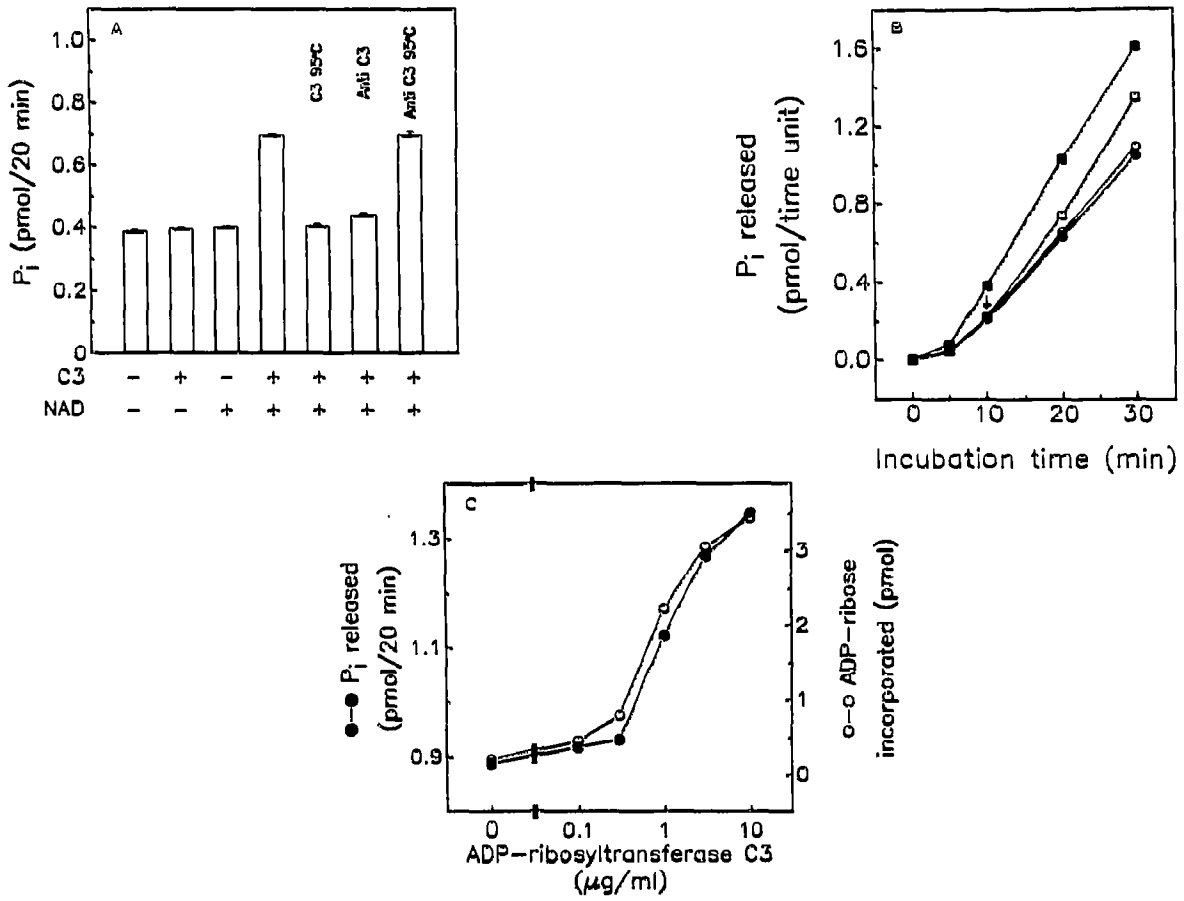

Fig. 1A. Influence of C3-induced ADP-ribosylation on GTP hydrolysis by recombinant rhoA. Recombinant rhoA (3 pmol [^{3}H]GTP-binding activity) was incubated in the absence and presence of 100 μM NAD without and with 0.1 μg C3 or heat-inactivated C3 (30 min, 95°C) for 10 min. Studies with anti-C3 antibody were performed after 60 min preincubation of C3 with the antibody or the heat-inactivated antibody (10 min, 95°C) at 0°C. Release of $^{32}P_i$ from [^{32}P]GTP was determined after further incubation for 20 min. Data are means (± S.D., n=3) of a representative experiment repeated three times. B. Effects of ADP-ribosylation on the time course of GTP hydrolysis by recombinant rhoA. rhoA was preincubated in the absence (○,●) and presence (□,■) of 0.1 μg C3 without (○,●) and with (■) 100 μM NAD for 10 min, then 50 nM [^{32}P]GTP (time point 0 min) was added and the $^{32}P_i$ release was determined at the indicated time points. The arrow indicates the addition of NAD at time point 10 min (□). Data are means of a representative experiment performed in duplicate and repeated three times. C. C3 concentration dependence of ADP-ribosylation and enhancement of GTP hydrolysis. ADP-ribosylation of recombinant rho (5 pmol [^{3}H]GTP-binding activity) was performed at the indicated concentrations of C3 in the presence of 10 μM [^{32}P]NAD or with unlabeled 10 μM NAD for 10 min. Thereafter, anti-C3 antibody was added to inhibit further ADP-ribosylation. Then, 50 nM [^{32}P]GTP was added for determination of GTP hydrolysis and the release of $^{32}P_i$ was determined after 20 min (●). The amount of incorporated [^{32}P]ADP-ribose (○) was studied by the filter assay. Data are means of a representative experiment performed in triplicate and repeated three times.

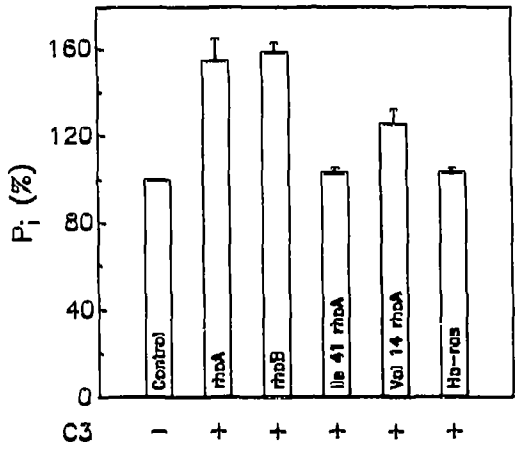

Fig. 2. Effects of ADP-ribosylation by C3 on the GTP hydrolysis by various GTP-binding proteins. rhoA, rhoB, Val-14 rhoA, Ile-41 rhoA and Ha-ras (each 3–10 pmol/tube) were ADP-ribosylated with 0.3 μg C3 and 100 μM NAD for 10 min. Then the release of $^{32}P_i$ from [^{32}P]GTP was determined after a further 20 min of incubation. Data are means ± S.E.M. of three independent experiments performed in triplicate.

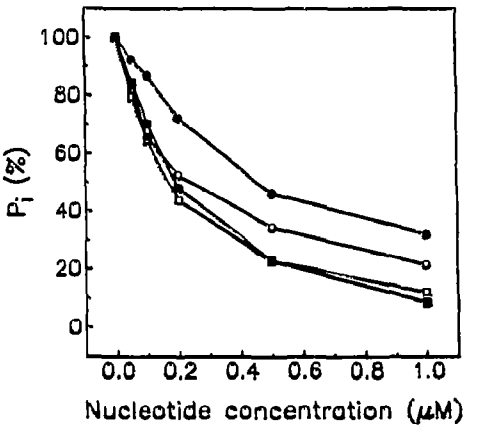

Fig. 3. Influence of ADP-ribosylation on [^{3}P]GTP hydrolysis in the presence of unlabeled GTP and GDP. rhoA was ADP-ribosylated in the absence (○,□) and presence (●,■) of 0.3 μg C3 and 100 μM NAD for 10 min at 30°C. Thereafter, the effects of increasing concentrations of unlabeled GTP (□,■) and GDP (○,●) on the hydrolysis of [^{32}P]GTP were studied. Data are means of a representative experiment performed in triplicate and repeated three times.

66 pmol [^{3}H]GTP (2 μCi; 30.4 Ci/mmol) for 10 min at 30°C in a total volume of 36 μl. Then triethanolamine-HCl and $MgCl_2$ were added to give 25 and 4.8 mM, respectively. ADP-ribosylation of preloaded rhoA was carried out in 30 mM triethanolamine HCl (pH 7.5), 1.7 mM $MgCl_2$, 1 mM dithiothreitol, 0.2 mM EDTA, 100 μg/ml bovine serum albumin, 0.5 mM phosphatidylcholine, 0.3 μg C3 without and with 100 μM NAD for 10 min at 30°C. Determination of the release reaction was started with the addition of 1 mM GTP. At the indicated periods of time 20 μl of the reaction mixture were transferred into 1 ml of washing buffer and immediately filtered through nitrocellulose membranes as described above.

3. RESULTS

The ADP-ribosylation of rhoA increased the steady-state GTP hydrolysis maximally by 50 to 80%. This effect depended on both the presence of the transferase (C3) and the co-substrate (NAD). Heat inactivation of C3 for 30 min at 95°C or pretreatment of C3 with a polyclonal anti-C3 antibody prevented the increase in [^{32}P]phosphate release (Fig. 1A). The time course of GTP hydrolysis caused by rhoA is depicted in Fig. 1B. After a short lag phase of about 5 min, which is most likely due to the slow GDP/[^{32}P]GTP exchange, the release of [^{32}P]phosphate was linear for at least 25 min. Prior ADP-ribosylation of rho protein with C3 and NAD increased the rate of phosphate release by about 50%. In the presence of C3 and without NAD no increase in GTP hydrolysis was detected. But the addition of NAD after 10 min increased the [^{32}P]phosphate release to rates observed with C3 and NAD present during the preincubation period. Fig. 1C shows the C3 concentration dependent ADP-ribosylation of rhoA and the increase in GTP hydrolysis. Both reactions occurred at the identical concentrations of the transferase suggesting a causal relationship of both phenomena. In order to test the specificity of the reaction, we studied the effect of C3-induced ADP-ribosylation on GTP hydrolysis with various rho proteins. As shown in Fig. 2, the ADP-ribosylation of recombinant rhoA and rhoB proteins increased GTP hydrolysis by about 50 and 60%, respectively. ADP-ribosylation of the Val-14 rhoA protein, a mutant which is characterized by a low endogenous GTPase activity, resulted in a small but consistently detected increase (about 20%) in [^{32}P]phosphate release. In contrast, GTP hydrolysis of Ile-41 rhoA was not increased after incubation with C3 and NAD. This protein lacks the ADP-ribose acceptor

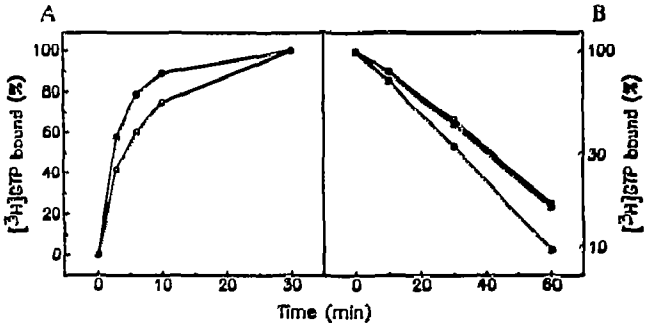

Fig. 4. Influence of ADP-ribosylation on the binding and release of [^{3}H]GTP/GDP from recombinant rhoA. A. rhoA protein (0.01 pmol) was ADP-ribosylated in the presence of 100 μM NAD without (○) and with (●) C3 (0.3 μg) for 10 min at 30°C. Thereafter, [^{3}H]GTP was added and binding was determined at the indicated time points. B. For determination of the release of [^{3}H]GTP/GDP rhoA was preloaded with [^{3}H]GTP by EDTA treatment and, thereafter, ADP-ribosylated with C3 (○), NAD (□) and C3 plus NAD (●) as described in the Method section. Thereafter, the release of [^{3}H]GDP was determined by measuring the remaining protein-bound radioactivity after addition of 1 mM unlabeled GTP by the filter assay. The free Mg^{2+} concentrations were 0.1 mM in the binding assay and 0.6 mM in the release assay. Data are means of a representative experiment performed in triplicate and repeated three times.

Asparagine-41 and cannot serve as a substrate for the transferase. Accordingly, the GTP hydrolysis catalyzed by Ha-ras was not affected by C3. This finding is in agreement with previous reports that Ha-ras is not a substrate for C3 [28].

Fig. 3 shows the influence of increasing concentrations of unlabeled GTP or GDP on hydrolysis of [^{3}H]GTP by recombinant rhoA. Whereas the isotope dilution curve of GTP was identical with control and ADP-ribosylated rhoA, a significant difference between unmodified and modified rhoA was found with increasing concentrations of GDP. Under control conditions the IC_{50} value for inhibition of [^{32}P]GTP hydrolysis was about 250 nM GDP. In contrast, with ADP-ribosylated rhoA GDP inhibited hydrolysis of [^{32}P]GTP half-maximally at about 450 nM.

Since the GTPase cycle of small GTP-binding proteins is most likely controlled by the slow release of bound GDP, we studied the influence of ADP-ribosylation on the release of previously bound [^{3}H]GTP. For this purpose, the exchange of rhoA-bound GDP with [^{3}H]GTP was accelerated by chelating Mg^{2+} in the presence of EDTA. Exchanged nucleotide was fixed by readdition of Mg^{2+}. Fig. 4B shows that the off-rate of bound nucleotide was increased after ADP-ribosylation. The half-life of bound [^{3}H]GTP/GDP was decreased from about 30 to 20 min at 0.6 mM free Mg^{2+}. Accordingly, a similar increase in the on-rate of [^{3}H]GTP binding was observed with ADP-ribosylated rhoA protein (Fig. 4A).

4. DISCUSSION

We studied the influence of the C3-induced ADP-ribosylation on the GTPase cycle of recombinant rho A protein by measuring the [^{32}P]phosphate released from the GTP-binding protein. In contrast to previous reports [23,24], we observed an about 50 to 80% increase in rho protein-catalyzed GTP hydrolysis in the presence of C3 and NAD. Several findings indicate that the increase in GTP hydrolysis was caused by ADP-ribosylation but not by any unspecific alteration of rho protein. Stimulation of GTP hydrolysis occurred only in the presence of C3 and NAD and not with either agent alone. The effect was blocked by anti-C3 antibody or heat treatment of the rather stable C3 for 30 min at 95°C. Moreover, the C3 concentration dependence for ADP-ribosylation of rho and increase in GTP hydrolysis correlated very well. Finally, the effect was observed with various rho proteins known to be substrates of C3. Thus, besides rhoA and rhoB even ADP-ribosylation of Val-14 rhoA, which is constitutively inhibited in its GTPase activity [6], resulted in a small but significant increase. In contrast, GTP hydrolysis by Ile-41 rhoA or Ha-ras protein which are no substrates for ADP-ribosylation was not increased.

The rate limiting step of the GTPase cycle of small GTP-binding proteins is suggested to be the release of bound GDP. Several findings indicate that the ADP-ribosylation does in fact facilitate the GDP/GTP exchange. For example, the inhibition curve of [^{32}P]GTP hydrolysis observed in the presence of increasing concentrations of GDP was shifted to higher GDP concentrations by ADP-ribosylation indicating a decrease in the affinity for GDP. Moreover, ADP-ribosylation increased the off-rate of the bound nucleotide.

Several other laboratories failed to observe an effect of ADP-ribosylation on GTP-binding or hydrolyzing activity of rho. So far, the reason for this discrepancy is not clear. We studied the influence of ADP-ribosylation on steady-state GTPase activity at rather low concentration of Mg^{2+} (100 μM), which increases GDP/GTP exchange and facilitates measurements of [^{32}P]phosphate release. Moreover, we used recombinant rho proteins which were purified by gel filtration and anion exchange chromatography only. Previous studies were performed with rho proteins purified from bovine brain preparations mostly by using detergents which may affect the GDP/GTP turn-over of the rho protein. Furthermore, recombinant rho differs from rho proteins purified from mammalian tissues in lacking the posttranslational modification, e.g. poly-isoprenylation at the C-terminus. ADP-ribosylation of rho occurs in Asparagine-41 which is located in the so-called effector region and rather distant from the C-terminus [3,4,22]. However, it is feasible that the missing posttranslational modification influences the overall structure of the rho protein and renders the GDP/GTP-binding more sensitive towards ADP-ribosylation.

It has been shown that the ADP-ribosylation does not block the interaction of the rhoA protein with its specific GTPase-activating protein (GAP) [12]. In consideration of the fact that GAPs increase the GTPase hydrolysis by small GTP-binding proteins several hundred-fold, the increase in GTP hydrolysis by ADP-ribosylation appears to be rather small. However, the data presented clearly indicate that ADP-ribosylation of rho protein affects the rate of the GTPase cycle. Further studies are necessary to clarify the functional consequences of the ADP-ribosylation on the activity state of rho and its interactions with the various regulating proteins like the guanine nucleotide dissociation stimulator (GDS) and dissociation inhibitor (GDI).

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