Purification of the 22 kDa protein substrate of botulinum ADP-ribosyltransferase C3 from porcine brain cytosol and its characterization as a GTP-binding protein highly homologous to the *rho* gene product

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The 22 kDa protein substrate of botulinum ADP-ribosyltransferase C3 was purified from porcine brain cytosol by acetone precipitation, CM-Sephadex, octyl-Sepharose and TSK phenyl-5PW HPLC chromatography to apparent homogeneity. ADP-ribosylation of the protein was increased by guanine nucleotides (GTP, GDP, GTPγS, each 100 μM) but not by GMP, ATP or ATPγS. The purified 22 kDa protein bound maximally 0.9 mol [^{35}S]GTPγS and hydrolyzed GTP with the rate 0.007 mol per mol protein. Amino acid sequences were obtained from two tryptic peptides, selected from an in situ digestion of Immobilon electrotransferred, gel purified ADP-ribosylated substrate. The two sequences obtained, cover 23 residues from the corresponding sequences in human *rho*.

ADP-ribosylation; ADP-ribosyltransferase C3; GTP-binding protein; GTP hydrolysis; Gene product, *rho*

1. INTRODUCTION

Beside botulinum C2 toxin, which ADP-ribosylates actin [1–4], certain strains of *Clostridium botulinum* Type C produce another ADP-ribosylating enzyme termed C3 [5,6]. Botulinum ADP-ribosyltransferase C3, which is structurally [7] and functionally [8] clearly distinct from botulinum neurotoxins C1 and D modifies cytosolic and/or membranous 22 to 24 kDa proteins in all eukaryotic tissues and cell types studied so far [5–9]. ADP-ribosylation by C3 is regulated by guanine nucleotides and divalent cations suggesting that the eukaryotic substrate of C3 is a GTP-binding protein [7,10]. Further support of this view was the observation that guanine nucleotides shielded the C3 substrate against heat inactivation [6,9]. These studies, however, did not exclude that guanine nucleotides regulate C3-induced ADP-ribosylation via an additional factor.

Here we report on the purification of the substrate of botulinum ADP-ribosyltransferase C3 from the cytosolic fraction of porcine brain tissue and its characterization as a GTP-binding protein, which is identical or highly homologous to the *rho* gene product.

2. MATERIALS AND METHODS

2.1. Materials

Botulinum ADP-ribosyltransferase C3 was purified as described [6]. Octyl-Sepharose and CM-Sephadex were purchased from Pharmacia (Freiburg, FRG). All nucleotides were obtained from Boehringer (Mannheim, FRG). [^{35}S]GTPγS and [γ-^{32}P]GTP were obtained from NEN (Dreieich, FRG). [^{32}P]NAD was prepared as described by Cassel and Pfeuffer [11] or obtained from NEN (Dreieich, FRG).

2.2. Tissue extraction

All procedures were performed at 4°C or as indicated. About 6 kg of porcine brain were homogenized in 10 l of PED buffer

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(25 mM potassium phosphate (pH 6.8), 3 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT and 0.2 mM PMSF) by using an Ultra Turrax. The extract was centrifuged in a Sorvall GSA rotor at 13000 rpm for 45 min. 0.8 vols of cold acetone (−20°C) were dropped to 1 vol. of the extract, while the mixture was stirred. The precipitation could occur overnight. The protein was sedimented by centrifugation at 13000 rpm for 15 min in a Sorvall GSA rotor. The precipitates were extracted with 1500 ml of PED buffer, again centrifuged and subjected to ion exchange chromatography on CM-Sephadex.

2.3. *CM-Sephadex column chromatography*

The column (10 × 50 cm) was equilibrated with PED buffer. The extract was applied to the column and approximately 1350 ml of the flow-through were collected.

2.4. *Octyl-Sepharose column chromatography*

NaCl (100 mM) and sodium cholate (0.3%, both final concentrations) were added to the flow-through of the CM-Sephadex column and the solution was applied to the column of octyl-Sepharose (5 × 14 cm) equilibrated with PED buffer containing 100 mM NaCl and 0.3% sodium cholate. The column was washed with 500 ml PED buffer containing 100 mM NaCl and 0.3% sodium cholate followed by a second wash with 500 ml PED buffer containing 500 mM NaCl and 0.3% sodium cholate. Proteins were eluted by a reversed gradient (2 × 500 ml) of NaCl (250–0 mM) and a gradient of sodium cholate (0.3–1.3%). The flow rate was approximately 50 ml/h and fractions of 7.5 ml were collected. The C3-substrate activity was eluted in a major peak. The fractions of the major peak were pooled and concentrated to about 2 ml by ultrafiltration with Amicon Dia-flow using PM 10 membranes and by Centriprep PM 10.

2.5. *Hydrophobic interaction chromatography on LKB TSK phenyl-5PW HPLC column*

The concentrated pool of the major peak of octyl-Sepharose chromatography was dialysed in 1 l PED buffer containing 250 mM NaCl and 0.1% sodium cholate. Thereafter, the protein was injected into a Waters HPLC, composed of a UK6 injector, a 6000 A pump and a Pye Unicam absorbance detector (LC-UV, 280 nm) equipped with LKB TSK phenyl-5PW (7.5 × 75 mm) HPLC column, previously equilibrated with PED buffer containing 250 mM NaCl and 0.05% sodium cholate. The column was washed with 11 ml of the equilibration buffer and bound protein was eluted by a reversed gradient of NaCl (250–0 mM) and a positive gradient of sodium cholate (0.05–1.3%). Time of gradient was 1 h and the gradient was stopped at 80% buffer B (PED buffer containing 1.3% sodium cholate). Fractions of 0.5 ml were collected and flow rate was 0.5 ml/min.

2.6. *ADP-ribosylation assay*

ADP-ribosylation was performed as described [1,5,6] in a medium containing 50 mM triethanolamine-HCl (pH 7.5), 0.5 mM ATP, 10 mM thymidine, 1 mM DTT, 10 μM GDP, 3 mM $MgCl_2$, 1 mM EDTA, 0.1 μM [^{32}P]NAD (about 0.8 μCi/tube) and 0.2 μg C3 or as indicated. Incubation was carried out for 30 min at 37°C in a total volume of 100 μl. For determination of maximal amount of protein modification ADP-ribosylation was performed in the presence of 50 μM NAD for 2 h. The reaction was terminated by addition of 400 μl of 0.1% bovine serum albumin, containing 2% SDS and 500 μl trichloroacetic acid (30%, w/v). Precipitated proteins were collected onto nitrocellulose filters. Filters were washed with 20 ml of 6% trichloroacetic acid and counted for radioactivity.

For SDS-polyacrylamide gel electrophoresis (SDS-PAGE) incubation was stopped by adding 900 μl trichloroacetic acid (20%, w/v). The pellet was washed with ether, dissolved in 50 μl sample buffer and subjected to 15% gel electrophoresis according to Laemmli [12]. Gels were stained with Coomassie blue destained and subjected to autoradiography for 10 to 24 h.

2.7. *GTPγS-binding assay*

GTPγS-binding was determined as described [13]. The binding buffer contained 6 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 800 mM NaCl, 0.1% Lubrol-PX, 1 μM [^{35}S]GTPγS (about 0.03 μCi/tube) and 20 mM Na-Hepes (pH 8.0). Incubation time was 120 min.

2.8. *GTPase activity assay*

GTP hydrolysis was determined as described [14] in a medium containing 0.5 μM [γ-^{32}P]GTP (about 0.1 μCi/tube), 1 mM $MgCl_2$, 1 mM DTT, 0.5 mM ATP, 50 mM triethanolamine-HCl (pH 7.5) and about 0.3 μg of purified C3-substrate protein.

2.9. *Amino acid sequence analysis*

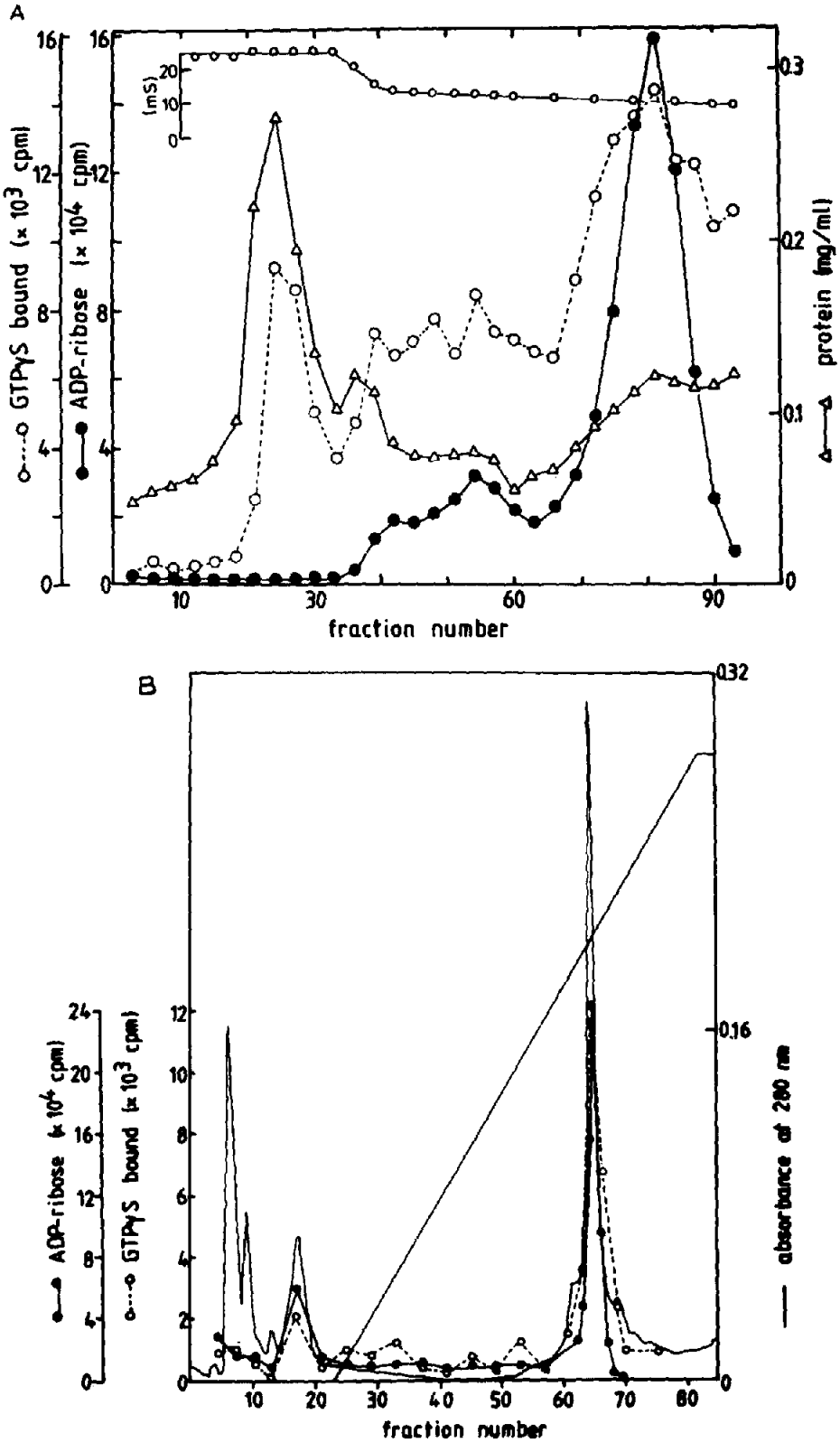
Approx. 10 μg of the C3 substrate-ADP-ribosyltransferase incubation mixture was purified on a 20% polyacrylamide gel and immediately electroblotted onto Immobilon membrane (Millipore). The electroblotting was carried out essentially as described [15], except that 50 mM triethanolamine/50 mM boric acid was used as transfer buffer [16]. Immobilized proteins were detected by staining with Amido Black. The in situ tryptic digestion was done as described by Bamo et al. [17] and the peptides eluting in the digestion medium were separated on a C4-reversed phase HPLC column (0.46–25 cm, 10 μm particle size, 300 A pore size; Vydac Separation Group, USA) equilibrated in 0.1% trifluoroacetic acid. Peptides were eluted with a linear gradient of increasing 70% acetonitrile, 0.1% trifluoroacetic acid applied over a period of 70 min and detected by adsorption at 214 nm wavelength. Peptide peaks were collected by hand in Eppendorf tubes and dried in a Speed Vac concentrator. The largest peaks were selected for amino acid sequence analysis. Therefore, we used a U70 A Applied Biosystems Inc. (USA) gas-phase sequenator, equipped with an on-line phenylthiohydantoin amino acid analyzer (model 120 A).

2.10. *Protein concentration*

Protein concentration was determined according to Bradford [18] with bovine serum albumin as standard.

3. RESULTS

In porcine brain the major substrate of botulinum ADP-ribosyltransferase C3 is an about 22 kDa cytosolic protein. Addition of cold acetone to the supernatant fraction of brain tissue


A
GTPγS bound (× 10³ cpm)
ADP-ribose (× 10⁴ cpm)
protein (mg/ml)
(mS)
fraction number
B
ADP-ribose (× 10⁴ cpm)
GTPγS bound (× 10³ cpm)
absorbance at 280 nm
fraction number

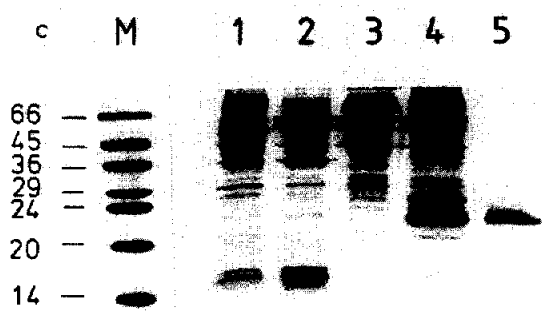


Fig.1. Purification of the substrate of botulinum ADP-ribosyltransferase C3. (A) Chromatography through octyl-Sepharose of the acetone precipitation fraction. The protein was eluted with a reversed gradient of NaCl (250–0 mM) and a gradient of sodium cholate (0.3–1.3%). Conductivity was measured (○—○) and the [^{35}S]GTPγS-binding (○---○) and [^{32}P]ADP-ribose incorporation (●—●) was determined in 20 μl aliquots of the indicated fractions. Protein (△—△) was determined according to Bradford [18]. (B) Chromatography on TSK phenyl-5PW HPLC column. The column was eluted with a reversed gradient of NaCl (250–0 mM) and a gradient of sodium cholate (0.05–1.3%). Protein was determined by absorbance at 280 nm (straight line). [^{35}S]GTPγS-binding (○---○) and [^{32}P]ADP-ribose incorporation (●—●) was determined in 30 μl aliquots of the fractions. (C) SDS-PAGE of the proteins of the various steps of purification. Lane M, marker; lane 1, cytosol (25 μg); lane 2, acetone precipitation (25 μg); lane 3, CM-Sephadex flow-through (25 μg); lane 4, octyl-Sepharose (25 μg); lane 5, TSK phenyl-5PW HPLC (3 μg).

precipitated the C3 substrate. When the dissolved protein pellet was subjected to cation exchange chromatography the 22 kDa C3 substrate was found in the flow-through. Thereafter, the flow-through was applied to octyl-Sepharose. Fig.1A shows that the C3 substrate eluted from octyl-Sepharose in one major peak, which coeluted with the major GTPγS binding activity. The active fractions of the major peak were pooled and subjected to LKB TSK phenyl-5PW HPLC column. From this column the C3 substrate eluted in one major peak, which again coeluted with the major GTPγS-binding activity (fig.1B). Fig.1C shows that the protein of this elution peak migrated as a single band on the SDS-gel with an apparent molecular mass of 22 kDa. The purification scheme of table 1 indicates that the C3 substrate was 4200-fold purified with an overall recovery of 2.3%. When the purified material was ADP-ribosylated under optimal conditions about 0.6 mol ADP-ribose per mol protein was incorporated in the substrate. As shown in fig.2, under these conditions the SDS-PAGE analysis revealed that the ADP-ribosylation of the substrate increased the relative molecular mass of the C3 substrate by about 1000 Da. Moreover, the SDS-PAGE indicates that more than 60% of the protein was ADP-ribosylated by C3. This discrepancy was most probably due to less specific C3-substrate activity determined by means of the filter assay.

At the various steps of the purification, the ADP-ribosylation of the 22 kDa protein by C3 largely depended on the presence of guanine nucleotides, which increased the ADP-ribosylation with the rank order GTPγS $\geq$ GDP > GTP > GDPβS, while GMP, ATP and ATPγS were without effects (not shown). However, stimulation of the ADP-ribosylation by guanine nucleotides

Table 1

Purification of the C3 substrate from porcine brain cytosol

	Volume (ml)	Protein (mg/ml)	Specific C3 substrate activity (pmol/mg)	Purification (-fold)	Recovery (%)
Extract	6360	15.4	6.5	1	100
Acetone precipitation	1420	9.2	15.9	2.4	33
CM-Sephadex	1350	3.8	34.2	5.3	28
Octyl-Sepharose	5	1.06	3125	480	2.6
TSK phenyl-5PW HPLC	2.5	0.211	27500	4230	2.3

At the various purification steps the C3 substrate activity was determined by ADP-ribosylation of an aliquot of the respective fraction in the presence of C3 (0.3 μg) and 50 μM [^{32}P]NAD

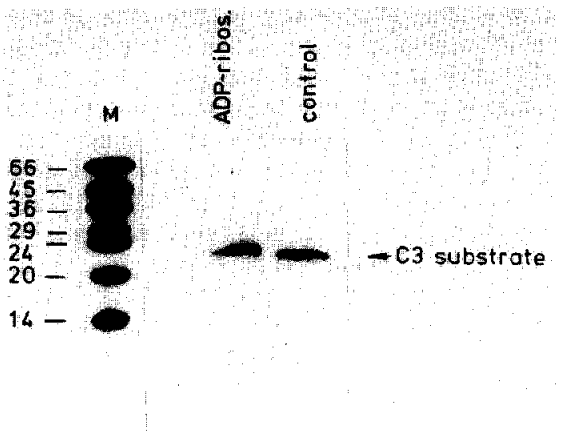


Fig.2. SDS-PAGE of the 22 kDa substrate of botulinum ADP-ribosyltransferase C3. 6 μg of the purified C3 substrate protein was ADP-ribosylated in the presence of 25 μM NAD without (control) and with 0.2 μg C3 (ADP-ribose) as described. About 2 μg of control and ADP-ribosylated proteins were analyzed by SDS-PAGE (15% polyacrylamide).

was almost completely prevented in the presence of Mg^{2+}, which itself largely increased the labeling of the 22 kDa protein.

The purified C3 substrate bound [^{35}S]GTPγS in a concentration dependent manner (fig.3A). About 0.9 mol GTPγS per mol C3 substrate was maximally bound. As GTP or GDP but not ATP (each 10 μM, not shown) competed for binding of [^{35}S]GTPγS (0.1 μM) to the C3 substrate, we studied whether a GTPase activity was associated with the GTP-binding substrate protein. Fig.3B shows that the 22 kDa protein released $^{32}P_i$ from [γ-^{32}P]GTP exhibiting a linear time course. The rate of GTP hydrolysis was about 7 mmol/min per mol C3 substrate.

In order to get more insight into the nature of the C3 substrate we partially analyzed the amino acid sequence of the C3 substrate. As the C3 substrate was blocked at the N-terminal end, we analyzed the three largest peaks obtained after tryptic digestion and separation by reversed phase

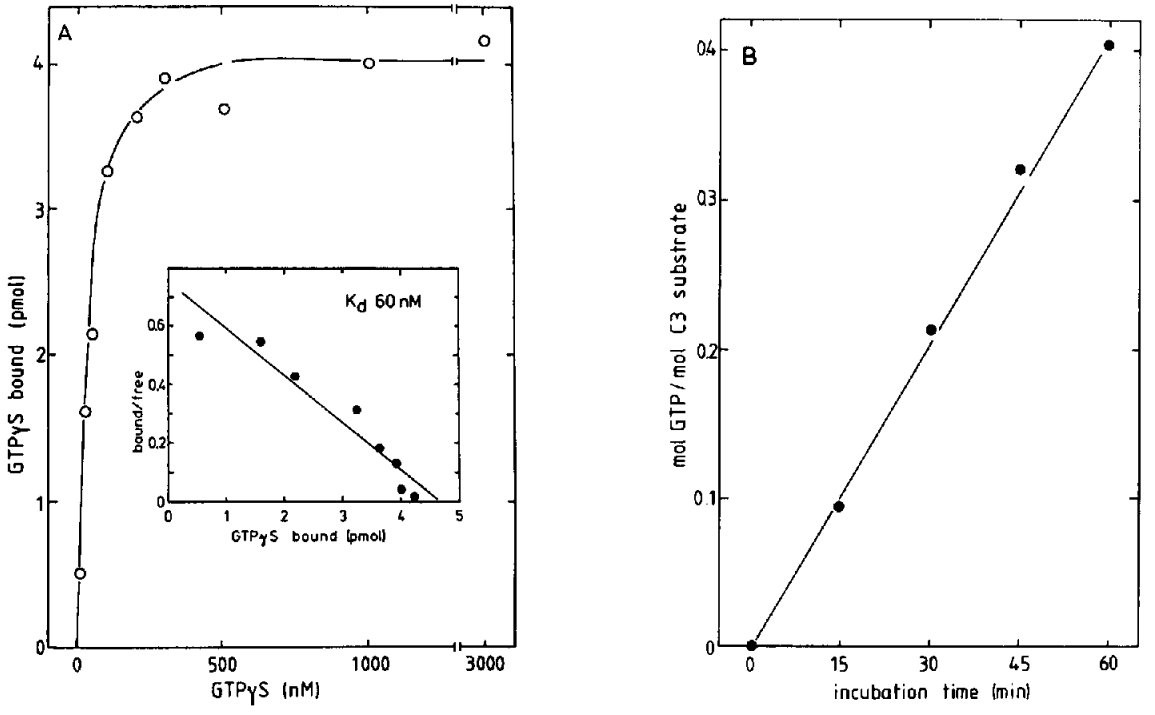


Fig.3. (A) Concentration dependency of [^{35}S]GTPγS-binding of the C3 substrate. Purified C3 substrate (0.12 μg) was incubated with the indicated concentrations of [^{35}S]GTPγS for 2 h at 30°C. Thereafter, bound nucleotide was determined as described. The inset shows the Scatchard plot. (B) Time course of GTP hydrolysis caused by the purified C3 substrate protein. Purified C3 substrate protein (0.3 μg) was incubated in the presence of 0.5 μM [γ-^{32}P]GTP for the indicated periods of time. Thereafter, $^{32}P_i$ released was determined as described.

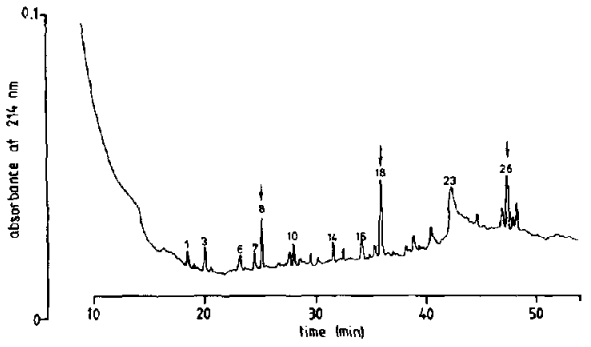


Fig.4. C4-reversed phase HPLC of the peptides generated by tryptic digestion of the C3 substrate. The C3 substrate was electroblotted onto an Immobilon membrane, in situ digested with trypsin and thereafter analyzed by HPLC. Peaks indicated by an arrow were applied to amino acid sequence analysis by a protein sequenator.

HPLC (fig.4). The sequence of peptide peaks 8 and 18 was: Trp-Thr-Pro-Glu-Val-Lys and Gln-Val-Glu-Leu-Ala-Leu-Trp-Asp-Thr-Ala-Gly-Gln-Glu-Asp-Tyr-Asp-Arg, respectively. Peak 26 did not yield any sequence. These sequences are identical to those covered by residues 99–104 and 52–68 respectively of human rho [19]. The limited sequence data demonstrate the high degree of structural homology with the *rho* proteins of other species and strongly suggest that the C3 substrate is the porcine *rho* protein or very similar to it, in line with its capacity to bind GTP.

4. DISCUSSION

We have purified the substrate of botulinum ADP-ribosyltransferase C3 from the cytosolic fraction of pig brain tissue to apparent homogeneity. This protein has an apparent molecular mass of 22 kDa, which is clearly increased after ADP-ribosylation. The protein binds GTPγS, which is competed by GDP and GTP but not by ATP and exhibits GTPase activity. Furthermore, the ADP-ribosylation of the purified protein is largely enhanced by guanine nucleotide in the absence of added Mg^{2+}. All these findings indicate that the substrate of C3 is indeed a GTP-binding protein. Moreover, amino acid sequence analysis of 2 peptides generated by tryptic digestion revealed that the substrate of C3 exhibits high homology to or is even identical with the bovine brain *rho* gene product. Recently, the purification of a *rho* gene product from bovine brain membranes has been reported [20]. This protein was shown to be a GTP-binding protein with a K_d value GTPγS of about 50 nM and exhibits a GTPase activity with a turnover number of about 0.01 min^{-1}. Thus, these data are in agreement with our results for the C3 substrate.

During the preparation of this manuscript the purification of a cytosolic protein from adrenal glands has been described [21], which is ADP-ribosylated by botulinum C1 neurotoxin. The protein from adrenal glands has a molecular mass of about 22 kDa and is apparently also a GTP-binding protein. Furthermore, from amino acid sequence data it appears that this protein is also highly homologous with the *rho* gene product (Narumiya, personal communication). In respect to our previous report that the ADP-ribosylation caused by botulinum neurotoxin C1 is caused by a contamination with C3 [7], it is feasible that the substrates from brain and adrenal glands are closely related.

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