Copurification of Rho Protein and the Rho–GDP Dissociation Inhibitor from Bovine Neutrophil Cytosol. Effect of Phosphoinositides on Rho ADP-Ribosylation by the C3 Exoenzyme of *Clostridium botulinum*†

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ABSTRACT: The substrate of the C3 exoenzyme from *botulinum* toxin is a protein which is particularly abundant in the cytosol of neutrophils [Stasia, M. J., Jouan, A., Bourmeyster, N., Boquet, P., & Vignais, P. V. (1991) *Biochem. Biophys. Res. Commun. 180,* 615–622]. Optimal conditions for the ADP-ribosylation of the C3 substrate have been established in order to follow the course of its purification from bovine neutrophil cytosol. In particular, phosphoinositides at micromolar concentrations were found to enhance the ADP-ribosylation capacity of the C3 substrate in crude neutrophil cytosol and partially purified fractions. A [^{32}P]ADP-ribosylatable protein, migrating on SDS–PAGE with a mass of 24 kDa, was copurified with a 29-kDa protein by a series of chromatographic steps on DEAE-Sephacel, Biogel P60, and Mono Q. In the case of the C3 substrate, isoelectric focusing revealed two major labeled bands with p*I* values of 6.2 and 5.6; the p*I* of the 29-kDa protein was 4.8–5.0. On the basis of the amino acid sequence of peptides resolved after proteolytic digestion, the 24-kDa protein and the 29-kDa protein were identified respectively as rho and the GDP dissociation inhibitor (GDI), suggesting that rho and GDI copurify from bovine neutrophil cytosol in the form of a complex. The presence of a number of amino acid residues specific of rho A in the enzymatic digest originating from rho indicates that, among the rho proteins, at least rho A belongs to the GDI–rho complex.

The rho proteins correspond to a class of small G proteins, which have the property to be ADP-ribosylated by the C3 exoenzyme of the *botulinum* toxin at an asparagine residue (Asn-41) (Sekine et al., 1989). They show 30% sequence identity with the ras proteins. Similarly to the ras proteins, the rho proteins have at their C terminus a conserved CAAX box, where C is cysteine, A's are two aliphatic amino acids, and X is any amino acid [for a review, cf. Macara (1991) and Valencia et al. (1991)]. The cysteinyl residue of the CAAX box is a site of prenylation after removal of the AAX sequence, and, in the case of the rho proteins, a geranyl-geranylation has been demonstrated (Yoshida et al., 1991). Like the ras proteins, the rho proteins exist under two interconvertible forms, an inactive GDP-bound form and an active GTP-bound form. The transition between these two forms is under the control of several proteins, including a GTPase activating protein (GAP) and two GDP/GTP exchange regulatory proteins, namely, a GDP dissociation stimulator (GDS) and a GDP dissociation inhibitor (GDI).[1] The rho proteins appear to control the organization and the functioning of the cytoskeleton, as shown by the striking changes caused by the C3 exoenzyme on the actin microfilament network in Vero cells (Chardin et al., 1989), in Swiss 3T3 cells (Paterson et al., 1990), and also in neutrophils (Stasia et al., 1991). Preliminary assays based on the C3-mediated ADP-ribosylation have shown that the rho proteins are preferentially localized in the cytosol and not in the membranes of neutrophil homogenates (Stasia et al., 1991). In bovine brain, rho B forms a soluble complex with GDI, and thereby the propensity of rho B to bind to the membrane by its hydrophobic geranyl-geranyl tail is inhibited (Yoshida et al., 1991). By analogy, it could be predicted that the presence of a soluble form of rho in neutrophils is due to its association with GDI to form a soluble complex. In this paper, we describe the copurification of rho and GDI, probably in the form of a complex, from bovine neutrophil cytosol, and the enhancing effect of dilution, detergents, and acidic lipids and most particularly phosphoinositides on the C3-mediated ADP-ribosylation of rho in crude cytosol and in partially purified fractions of rho.

EXPERIMENTAL PROCEDURES

Materials. The following materials were supplied by the companies indicated: [^{32}P]NAD by NEN; diisopropyl fluorophosphate (DFP), Mops, DTT, EGTA, Hepes, thymidine, isonicotinic hydrazine, sodium deoxycholate, fatty acids, and phospholipids by Sigma Chemical Co.; β-mercaptoethanol, SDS, Coomassie brillant Blue, and bromophenol blue by Serva; silver nitrate by Prolabo; acrylamide and bis(acrylamide) by BDH Biochemicals; DEAE-Sephacel, Mono QHR 5/5, and standard molecular weight markers by LKB Pharmacia; Biogel P60 by Bio-Rad; trypsin and Asp endoprotease by Boehringer. The C3 ADP-ribosyltransferase was obtained from *Escherichia coli* transformed with a plasmid in which the cloned gene of exoenzyme C3 was inserted (Popoff et al., 1991). C3 was produced in *E. coli* in the cytoplasma after induction by

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[1] Abbreviations: exoenzyme C3, *Clostridium botulinum* ADP-ribosyltransferase C3; GDI, GDP dissociation inhibitor; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DFP, diisopropyl fluorophosphate; PIP, phosphatidylinositol monophosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; DOC, sodium deoxycholate; IPTG, isopropyl thiogalactoside; DTT, dithiothreitol.

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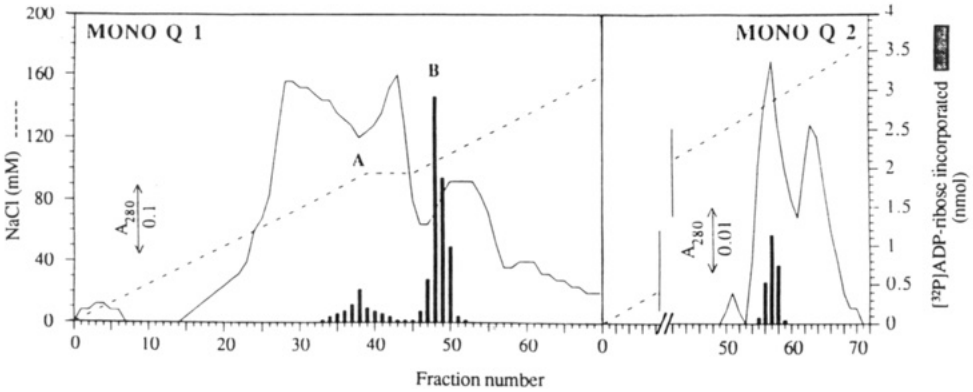


FIGURE 1: Chromatographic fractionation of the C3 substrate on Mono Q. The active fraction recovered from Biogel P-60 (61 mg of protein) was chromatographed on Mono Q. Two sequential chromatographies on Mono Q were performed as described under Experimental Procedures. Pool B (3 mg of protein) from the first Mono Q chromatography (Mono Q1) was subjected to an additional chromatography on Mono Q (Mono Q2). The C3 substrate in the eluate (bars) was assessed by ADP-ribosylation with [^{32}P]NAD and the C3 exoenzyme (cf. Experimental Procedures). The data are expressed as picomoles of bound [^{32}P]ADP-ribose present in the eluates.

IPTG. After disruption of the bacterial membrane by three cycles of freeze–thawing in 20 mM Hepes buffer, pH 8, the C3 exoenzyme was purified by affinity chromatography on bound anti-C3 polyclonal IgG.

Protein Assays. Protein concentration was determined by the method of Bradford (1976). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to the method described by Laemmli and Favre (1975), with a 4% stacking gel and a 13% resolving gel. Two-dimensional gel electrophoresis was performed by the method of O'Farrell (1975), with slight modifications (Stasia et al., 1989). For determination of the isoelectric point (p*I*), only the first dimension was used. The pH gradient was determined by cutting the gel into 5-mm slices. Each slice was soaked in degassed and deionized water, and the pH was measured. Silver staining was performed as described by Wray et al. (1981).

Assay of C3 Substrate. Fractions recovered during the purification procedure were assayed for their ability to be ADP-ribosylated by the C3 exoenzyme from *Clostridium botulinum*, using the procedure described by Chardin et al. (1989) after minor modifications. Briefly, 5–10 μL of the purified fractions was mixed with 20 mM Hepes, pH 7.8, in the presence of 15 mM isonicotinic acid, 15 mM thymidine, 2.5 mM $MgCl_2$, 1 mM ATP, and 10 ng of C3. The reaction was started with the addition of 3.5 μM [^{32}P]NAD (20 000–30 000 dpm/pmol). The final volume was 20 μL. Incubation was performed at 37 °C for 1 h. It was stopped by addition of 10 μL of a solution consisting of 15% (v/v) glycerol, 2.3% SDS, 0.001% bromophenol blue, 0.06 M Tris-HCl, pH 6.8, and 5% mercaptoethanol. After concentration under vacuum to 15 μL, the samples were subjected to SDS–PAGE. An autoradiogram was made, using Amersham X-ray film. For quantification of the amount of bound [^{32}P]ADP-ribose, the portions of the gel corresponding to the labeled bands were cut out, and the radioactivity was measured by liquid scintillation counting.

Fractionation of Bovine Neutrophils. Bovine neutrophils were routinely prepared from 10 L of blood, as described by Morel et al. (1985). Neutrophils were suspended in 20 mM Mops buffer, pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA, and 1 mM DTT, supplemented with 1 mM DFP. After a 10-min incubation at 4 °C, they were disrupted by ultrasonic treatment for 3 × 15 s with a Branson sonifier at 40-W output, and the homogenate was centrifuged at 140000*g* for 1 h. The supernatant, referred to as cytosol, was used for purification of the C3 substrate.

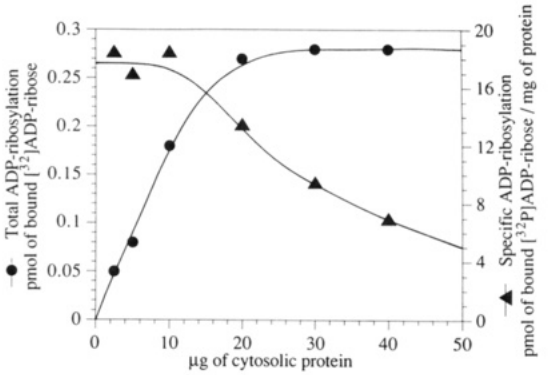


FIGURE 2: Effect of the amount of cytosolic protein of total ADP-ribosylation and specific ADP-ribosylation. Conditions of [^{32}P]ADP-ribosylation are described under Experimental Procedures. The amount of cytosolic protein was between 2.5 and 40 μg for a final volume of 20 μL. Note that the level of [^{32}P]ADP-ribose incorporated in the C3 substrate does not increase with increasing amounts of cytosolic protein above a threshold value of 10–15 μg of protein.

Purification of the C3 Substrate from Bovine Neutrophil Cytosol. A volume of 50 mL of bovine neutrophil cytosol corresponding to 800–900 mg of protein was applied to a DEAE-Sephacel (Pharmacia) column (8.8 cm × 2.9 cm) equilibrated with buffer A (20 mM Mops, pH 7.4, 1 mM EGTA, and 1 mM DTT). The column was washed with 200 mL of the equilibration buffer and eluted with a linear gradient of NaCl (0–200 mM) in 300 mL of buffer A at a flow rate of 30 mL/h. The eluate was collected in fractions of 5 mL. The fractions eluted between 90 and 110 mM NaCl contained the C3 substrate. They were pooled, concentrated to 0.5 mL by ultrafiltration on a Centripep 10, and then chromatographed on a Biogel P-60 column (70 cm × 1.6 cm) equilibrated in buffer A supplemented with 100 mM NaCl and 0.03% sodium deoxycholate using the same solution for elution. The C3 substrate was recovered, in fractions of 2.4 mL, between 40 and 50 mL of the eluted volume. The active fractions (8–10 mL) were pooled, and the volume was diluted to 50 mL by addition of buffer B consisting of 20 mM Tris, 1 mM EGTA, and 1 mM DTT, final pH 7.4. This was chromatographed on a Mono QHR 5/5 column equilibrated in buffer B (Figure 1). The column was washed with 15 mL of this buffer and then eluted by fractions of 0.5 mL with 40 mL of a linear gradient of NaCl (0–200 mM) in buffer B. Some C3 substrate was eluted between 80 and 95 mM NaCl (pool A). When the salt molarity reached 95 mM, the gradient was stopped for

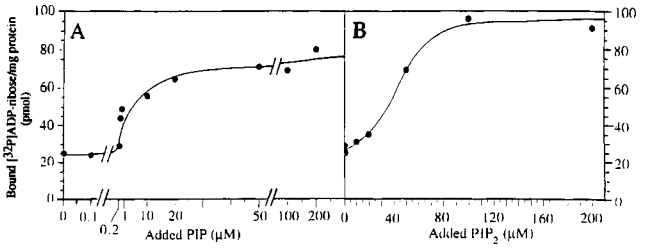


FIGURE 3: Effect of increasing concentrations of phosphatidylinositol phosphate and phosphatidylinositol 4,5-bisphosphate on specific ADP-ribosylation of bovine neutrophil cytosol (bound ADP-ribose per milligrams of protein). The assay conditions are described under Experimental Procedures. (A) Phosphatidylinositol phosphate (PIP); (B) phosphatidylinositol 4,5-bisphosphate (PIP_2). ADP-Ribosylation was carried out with an amount of crude neutrophil cytosol corresponding to 13 μg of protein, the final volume of the incubation medium being 20 μL.

5 min and then run again. This procedure allowed the further elution, between 95 and 110 mM NaCl, of a significant amount of C3 substrate in a rather purified form in only 1.5–2.0 mL of the saline solution (pool B). The C3 substrate in pool B was found by SDS–PAGE to be devoid of a 23-kDa Ca^{2+} binding protein that was present at high concentration (15–20%) in the cytosol of bovine neutrophil (Dianoux et al., 1992) and that comigrated with the C3 substrate in pool A. Pool B recovered from this first Mono Q column was diluted to a final volume of 50 mL by addition of buffer B with the pH adjusted to 8.2 (buffer C), and applied to a second Mono QHR 5/5 column equilibrated in buffer C. This column was washed with 10 mL of buffer C and eluted with 40 mL of a linear gradient of NaCl (0–200 mM) in buffer C at a flow rate of 0.5 mL/min. The C3 substrate was recovered in about 1.5 mL of eluate with an NaCl concentration of 125–150 mM. This purified fraction was termed Mono Q2 (Figure 1).

Reverse-Phase HPLC of the Cleavage Fragments and Peptide Sequence Analysis. Following SDS–PAGE, the gel was slightly stained for protein with 0.1% Coomassie Blue R-250 in 50% methanol and 10% acetic acid for 5 min and destained with 50% methanol and 10% acetic acid for 10 min at room temperature. Two protein bands of 24 and 29 kDa, which were the only ones present at the last step of the purification, were excised and subjected to the following treatment (Swanson et al., 1991). First, the SDS was extracted twice with 20 mL of 50% methanol for 10–15 min. Each slice of gel was soaked in 20 mL of 100 mM ammonium bicarbonate, pH 8.2 (for tryptic treatment), or in 50 mM phosphate buffer, pH 8.0 (for endo-Asp treatment), for 15 min, then placed in an Eppendorf tube to be moderately dried under vacuum, and finally crushed with the pestle of a miniPotter. Five hundred microliters of ammonium bicarbonate or phosphate buffer was added, followed by trypsin or Asp endoprotease, respectively, using an enzyme to protein ratio of about 1/50. Incubation was carried out overnight at 37 °C in the presence of 5% acetonitrile. The soluble fraction was recovered after filtration through a Spin-X (0.45 μm) filter. Then the gel was rinsed in the cleavage buffer. The soluble extracts were concentrated to about 100 μL under vacuum and filtered through a Spin-X (0.45 μm) filter, and the peptides were separated by reverse-phase high-performance chromatography (HPLC), using a VYDAC TP C4 column (5 μm, 300 Å, 250 × 2.1 mm) in 0.1% trifluoroacetic acid, and eluted at a flow rate of 300 μL/min with a gradient from 0% to 80% acetonitrile supplemented with 0.1% trifluoroacetic acid. Peptide-containing fractions were collected manually in Eppendorf tubes, based on their UV absorption at 215 nm. The collected fractions were spotted onto a glass fiber disk coated with polybrene, and amino acid sequence analysis was performed on an automated Applied Biosystems gas-phase sequenator (477A) equipped with an on-line PTH-amino acid analyzer (Model 120A).

Table I: Effect of Lipids and Detergents on the ADP-Ribosylation Capacity of Neutrophil Cytosol[a]

expt	added ligand (mM)	ADP-ribosylation capacity (pmol/mg of cytosolic protein)
1	none	30
	arachidonic acid (0.5)	140
	oleic acid (0.1)	120
	palmitoleic acid (0.1)	40
	myristoleic acid (0.1)	30
2	none	35
	arachidic acid (1)	100
	stearic acid (1)	70
	palmitic acid (1)	55
	myristic acid (1)	40
	lauric acid (1)	40
3	none	30
	phosphatidylinositol monophosphate (0.2)	80
	phosphatidylinositol bisphosphate (0.2)	95
	phosphatidylserine (0.2)	60
	phosphatidylethanolamine (0.2)	55
4	none	32
	cardiolipin (0.2)	95
	sodium deoxycholate (1.0)	95
	sodium dodecyl sulfate (1.0)	105

[a] The experimental conditions are described under Experimental Procedures. All the lipids used in experiments 1 and 2, plus phosphatidylserine, phosphatidylethanolamine, and cardiolipin in experiments 3 and 4, were in the form of liposomes prepared as follows. A solution of lipid (5–10 mg) in chloroform was dried under a flow of nitrogen in a glass tube. The thin layer of lipid coating the tube was taken up in 1 mL of 0.12 M KCl and sonicated for 30 s with a sonicator probe. Phosphatidylinositol monophosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2) were directly suspended in 0.12 M KCl. The amount of cytosolic protein used per test was 12–15 μg in a final volume of 20 μL. The concentrations of ligands reported in the table are those found to enhance maximally the ADP-ribose uptake. In the case of PIP and PIP_2, a large plateau of optimal concentration was found (cf. Figure 3). Inositol and inositol 1,4,5-trisphosphate (IP_3) tested in the above system were unable to enhance the ADP-ribosylation capacity of neutrophil cytosol.

RESULTS

Optimal Conditions for Assay of Rho Protein by ADP-Ribosylation with Exoenzyme C3. An accurate assessment of the ADP-ribosylation capacity of the C3 substrate (rho protein) requires adjustment of the amount of protein used in the ADP-ribosylation assay. This is illustrated in the experiment shown in Figure 2, in which the total capacity of ADP-ribosylation of crude cytosol (total [^{32}P]ADP-ribose bound) and its specific ADP-ribosylation capacity ([^{32}P]ADP-ribose bound/mg of protein) were assessed as a function of the amount of cytosol used, in the assay. Above a threshold amount of 10–15 μg of cytosolic protein, the total amount of [^{32}P]ADP-ribose bound did not increase, and so the amount of [^{32}P]ADP-ribose bound per milligram of protein diminished. Such behavior is encountered in the case of enzymes bound to natural inhibitors. In fact, the assayed enzymatic activity is enhanced upon dilution, due to the release of the bound inhibitor from the enzyme–inhibitor complex. By analogy, in the case of the C3 substrate, it is inferred that the interaction between the C3 exoenzyme and its substrate, namely, a rho protein, is impeded by the formation of a complex between the C3 substrate and a putative inhibitor present in cytosol; thus, dilution would favor the release of the bound inhibitor and increase the ADP-ribosylation capacity of the C3

Table II: Purification of the C3 Substrate from Bovine Neutrophil Cytosol[a]

step	fractions	protein total (mg)	total binding capacity for [^{32}P]ADP-ribose (pmol)	specific binding capacity for [^{32}P]ADP-ribose (pmol/mg of protein)	purification (x-fold)
I	cytosol	884	23630 (38900)[b]	27 (44)[b]	1
II	DEAE-Sephacel	140	11900 (27860)	85 (198)	3.2
III	Biogel P-60	61	8170 (18140)	134 (297)	5.0
IV	Mono Q1 (pool A)	7.2	1610 (1440)	223 (200)	8.3
IV	Mono Q1 (pool B)	3.0	6370 (8580)	2123 (2860)	79
V	Mono Q2[c]	0.1	2430 (2020)	24300 (20200)	900

[a] Conditions of assays are given under Experimental Procedures. [b] Values in parentheses correspond to the total of specific ADP-ribosylation assayed in the presence of 0.1 mM PIP. At the steps of Mono Q1 and Mono Q2, similar values were obtained with or without added PIP. [c] Only pool B from Mono Q1 was purified on Mono Q2.

substrate. This dilution effect was observed in neutrophil cytosol and in partially purified fractions from cytosol.

In this context, it is interesting to note that the C3-dependent ADP-ribosylation of brain extracts was reported to be enhanced by detergents and some phospholipids (Maehama et al., 1990; Williamson et al., 1990). We have been able to confirm these data and quantify some of them in the case of bovine neutrophil cytosol. In Table I are compiled data concerning the effect of a number of saturated and unsaturated fatty acids, phospholipids, and detergents used at optimal concentrations. An enhancement of the ADP-ribosylation capacity, ranging between 2- and 4-fold, was found with 0.1–1 mM arachidonic acid, oleic acid, arachidic acid, stearic acid, palmitic acid, PIP, PIP_2, phosphatidylserine, phosphatidylethanolamine, cardiolipin, SDS, and DOC. The effect of fatty acids depended on the chain length. It was significantly higher with long-chain fatty acids and, for the same chain length, with the degree of unsaturation. The dose effect curves relative to the enhancing effect of PIP and PIP_2 on the ADP-ribosylation activity of crude neutrophil cytosol are illustrated in Figure 3. With PIP, maximal stimulation was achieved with 20 μM, whereas PIP_2 showed the maximal enhancing effect above 80 μM. In both cases, the curves were sigmoidal, with an abrupt increase in ADP-ribose uptake above a threshold concentration of 0.2 μM in the case of PIP and 20 μM in that of PIP_2. The significance of this sigmoidal profile is presently difficult to interpret. A trivial possibility might be that a number of cytosolic proteins are able to bind PIP and PIP_2 with high affinity. The binding of PIP or PIP_2 to these high-affinity proteins might prevail on the binding to specific protein(s) responsible for the enhancement of the ADP-ribose uptake. It should be noted that the rate of ADP-ribosylation was much less affected than the plateau of ADP-ribosylation. Enhancement of the ADP-ribosylation capacity of the C3 substrate by phosphoinositides was found in neutrophil cytosol, and in the partially purified fractions from DEAE-Sephacel, and Biogel P60, but was barely detectable in the Mono Q purified fraction, as evidenced from the amounts of ADP-ribose taken up by active fractions in the absence and presence of PIP at different steps of the purification procedure (Table II).

Copurification of the C3 Substrate (Rho Protein) and GDI from Bovine Neutrophil Cytosol. A summary of a typical purification of the C3 substrate assayed by [^{32}P]ADP-ribosylation is shown in Table II. In the absence of added PIP, the calculated purification factor at the Mono Q2 step was 900. When the ADP-ribosylation assay was performed in the presence of 100 μM PIP, a purification factor of about 460 was found. This is explained by the important enhancing effect of PIP on the ADP-ribosylation capacity of cytosol which contrasts with a lack of effect of PIP on the purified fraction. The presence of 0.03% deoxycholate in the elution

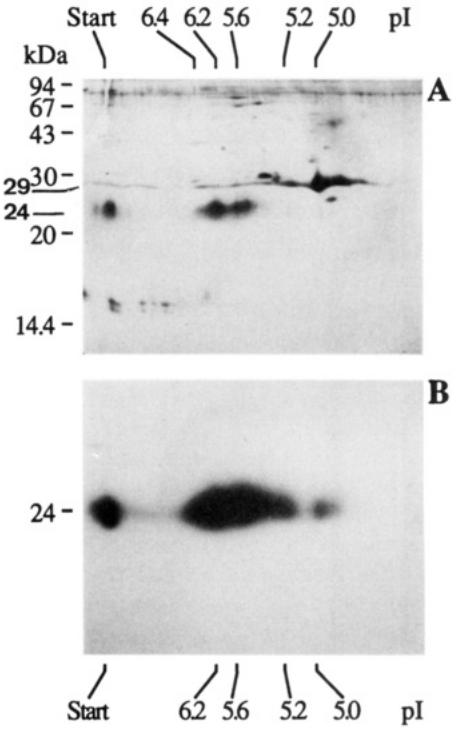


FIGURE 4: 2D-PAGE of the purified protein fraction recovered from the last step (Mono Q2) of purification of the C3 substrate from bovine neutrophil cytosol. (A) The purified fraction from the second Mono Q (Mono Q2) column was [^{32}P]ADP-ribosylated by exoenzyme C3 and then subjected first to isoelectric focusing and then to SDS–PAGE. The gel was stained with silver nitrate. (B) Autoradiogram of the gel showing two major labeled spots at pH 6.2 and 5.6 and two minor ones at pH 5.2 and 5.0.

buffer for chromatography on the Biogel P-60 column markedly improved the resolution of the C3 substrate. It allowed the recovery of the C3 substrate in a narrow peak corresponding to a molecular mass of 45 kDa. In the absence of deoxycholate, the peak of elution was much broader with a large tail, suggesting some retention. A more serious effect of the omission of deoxycholate was the contamination of an abundant Ca^{2+} binding protein found in the cytosol of bovine neutrophil amounting to 15–20% of the proteins of cytosol (Stasia et al., 1989; Dianoux et al., 1992).

The procedure adopted for the elution of the C3 substrate from the first column of Mono Q, consisting of interrupting the NaCl gradient for a few minutes and then resuming the flow of the NaCl solution, facilitated the recovery of C3 substrate still bound to Mono Q in a very narrow concentration range of the NaCl gradient (95–110 mM) (Figure 1). This fraction was further purified by an additional chromatography step on a second column of Mono Q. The active eluate, termed Mono Q2, was found to contain the C3 substrate in a high degree of purity and essentially associated with only one protein

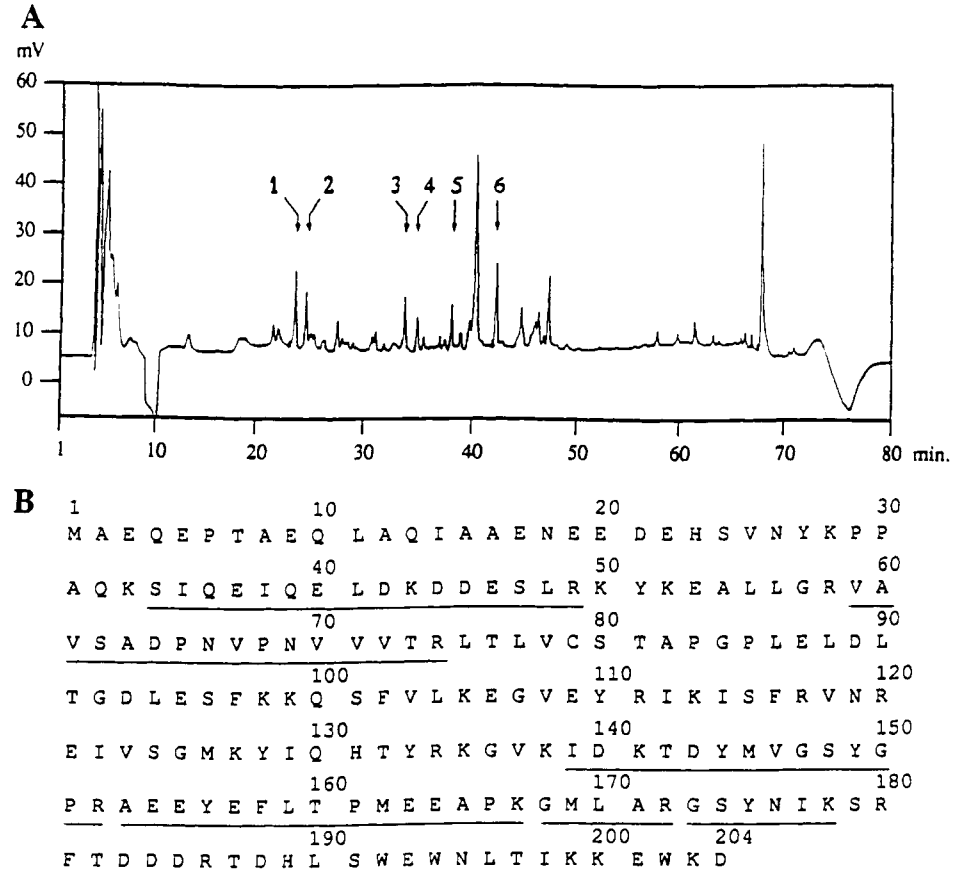


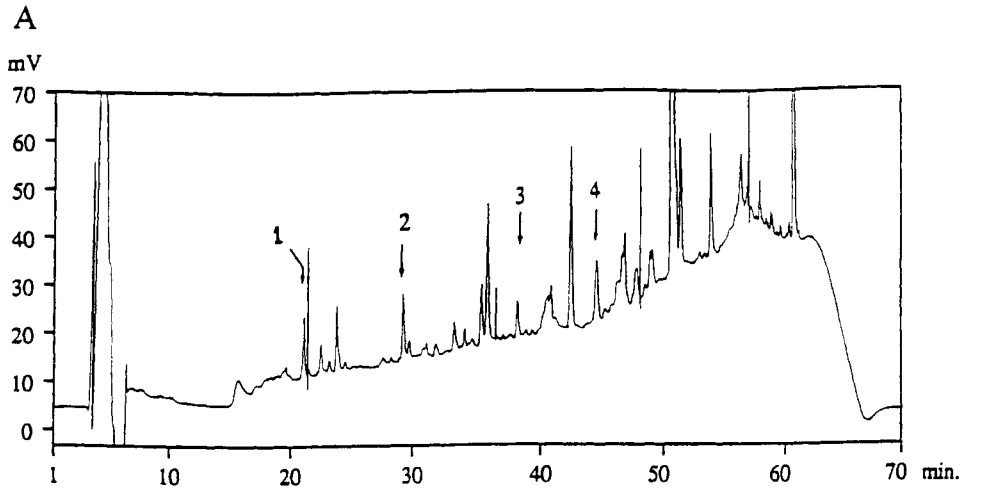
FIGURE 5: (A) Elution pattern of a tryptic digest of the 29-kDa protein copurified with rho from bovine neutrophil cytosol. Conditions for chromatographic resolution of the tryptic peptides on reverse-phase HPLC are described under Experimental Procedures. (B) Comparison of the sequence of tryptic peptides recovered from HPLC with the sequence of GDI deduced from the corresponding cDNA (Fukumoto et al., 1990). Identical sequences are underlined. The correspondence between peaks of the elution pattern of the tryptic digest (A) and the sequences (B) is as follows: peak 1, G173–K178; peak 2, G168–R172; peak 3, I139–R152; peak 4, V59–R74; peak 5, S34–R49; peak 6, A153–K167.

that was identified as GDI (see below). The protocol of preparation of the C3 substrate–GDI complex described here was tested with four different bovine neutrophil extracts. In all cases, a C3-substrate–GDI heterodimer was isolated.

Physical Properties of the Protein Complex Recovered from Mono Q. The apparent molecular mass of the active C3 substrate assessed by filtration on Biogel P60 was around 45 kDa, suggesting that, if the C3 substrate is a rho protein of 24 kDa, it is eluted as a complex with another protein of similar molecular mass. This putative complex was recovered in a homogeneous form at the step of the Mono Q2 chromatography. It was resolved by 2D PAGE (Figure 4). Two intensely labeled [^{32}P]ADP-ribosylated bands with p*I*'s of 6.2 and 5.6, and two minor labeled bands with p*I*'s of 5.2 and 5.0, corresponding to the C3 substrate, were visualized on the autoradiogram (Figure 4B), and were found to coincide with the silver nitrate stained bands on the gel (Figure 4A). In SDS–PAGE, all of the above labeled bands migrated with an apparent molecular mass of 24 kDa. This labeling pattern was fully reproducible. It poses the question of whether the same protein can contain multiple ADP-ribosylations, although this is not a conventional view, and that the calculated amount of [^{32}P]ADP-ribose incorporated at the last step of the purification, expressed as moles per mole of the 24-kDa protein, was roughly 0.6.

In addition to the [^{32}P]ADP-ribosylated bands, a nonlabeled band with a p*I* of 4.8–5.0, and an apparent molecular mass of 29 kD, was revealed by silver nitrate staining (Figure 4A).

Partial Amino Acid Sequence of the ADP-Ribosylated 24-kDa Protein and the 29-kDa Protein. As detailed under Experimental Procedures, the 29- and 24-kDa bands migrating on SDS–PAGE were cut out of the gel and subjected to digestion with trypsin and endo-Asp protease, respectively. The digests were fractionated by reverse-phase HPLC. In the case of the 29-kDa protein, the sequences of several tryptic peptides separated by HPLC were found to be identical with segments of the cDNA-deduced amino acid sequences of rho–GDI (Fukumoto et al., 1990). As shown in Figure 5, these sequences extended from Ser-34 to Arg-49, from Val-59 to Arg-74, from Ile-139 to Arg-152, from Ala-153 to Lys-167, from Gly-168 to Arg-172, and from Gly-173 to Lys-178. In the case of the 24-kDa protein, four amino acid sequences corresponding to peptide fragments generated by cleavage with the endo-Asp protease were examined and found to be related to rho (Figure 6). Two of them extending from Asp-45 to Trp-58 and from Asp-67 to Pro-75 were identical with sequences common to rho A, B, and C. The other two fragments, Glu-130–Arg-145 and Asp-28–Ala-44, corresponded to sequences specific of rho A (Yeramian et al., 1987). Sequences specific of rho B and rho C were not detected in the fractions analyzed, but their presence cannot be excluded in other fractions. These results led us to conclude that the 29-kDa protein and the 24-kDa protein are GDI and rho, respectively, and that, among the rho proteins, at least rho A is present at the last step of the purification. Taken together with the fact that these two proteins are recovered in the same fraction after several steps of chromatography (Table II), it is likely that the two neutrophil cytosolic proteins, rho A and GDI, are associated in a complex.



B

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          10                  20                  30
M A A I R K K L V I  V G D G A C G K T C  L L I V F S K D Q F
          40                  50                  60
P E V Y V P T V F E  N Y V A D I E V D G  K Q V E L A L W D T
          70                  80                  90
A G Q E D Y D R L R  P L S Y P D T D V I  L M C F S I D S P D
          100                 110                 120
S L E N I P E K W T  P E V K H F C P N V  P I I L V G N K K D
          130                 140                 150
L R N D E H T R R E  L A K M K Q E P V K  P E E G R D M A N R
          160                 170                 180
I G A F G Y M E C S  A K T K D G V R E V  F E M A T R A A L Q
          190
A R R G K K K S G C  L V L
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FIGURE 6: (A) Elution pattern of an endo-Asp protease digest of the 24-kDa protein copurified with GDI from bovine neutrophil cytosol. Conditions for the separation of the peptides by reverse-phase HPLC are described under Experimental Procedures. (B) Comparison of the sequence of the peptides of the endo-Asp protease digest recovered from HPLC with the sequence of rho A deduced from the corresponding cDNA (Yeramian et al., 1987). Identical sequences are underlined. Two sequences, D45–W58 (peak 3 in the elution pattern) and D67–P75 (peak 2), are common to rho A, B, and C. Two other sequences, D28–A44 (peak 4) and E130–R145 (peak 1), contain amino acid residues (boldface letters) that are totally or partially specific of rho A. The cleavage at the level of E130 by the endo-Asp protease might be explained by a protease contaminant present in a minor amount or by a slightly different specificity of attack of the enzyme due to a peculiar sequence of amino acid residues around E130.

DISCUSSION

In a previous study, it was shown that rho protein is abundantly present in neutrophils, and is predominantly located in the cytosol (Stasia et al., 1991). Rho is geranyl-geranylated (Yoshida et al., 1991) and has therefore a propensity to bind to the membrane by its geranyl-geranyl hydrophobic tail. To explain the fact that rho and other small G proteins are found not only in membranes but also in the cytosol, it has been postulated that the cytosolic soluble form of rho is the result of the formation of a complex between rho and the GDP dissociation inhibitor protein (GDI) (Ueda et al., 1990; Fukomoto et al., 1990; Isomura et al., 1991). It was hypothesized that GDI inhibits the GDP/GTP exchange reaction on rho by inhibiting the dissociation of GDP, and by preventing the subsequent binding of GTP (Hori et al., 1991). It was predicted that when GDI is dissociated from the GDP-bound form of rho, then rho binds to the membrane and is converted to its GTP-bound form (Isomura et al., 1991). GDI is abundantly present in a number of rat tissues including cerebrum (mainly cytosol), cerebellum, thymus, lung, small intestine, and spleen, but is present in limited amounts in heart and skeletal muscle (Shimuzu et al., 1991). Our results show that bovine neutrophil cytosol is also a source of GDI. In addition, the occurrence of an association between GDI and rho in bovine neutrophil cytosol is strongly suggested by the fact that, at the ultimate step of the purification of rho, GDI is the only protein recovered with rho in the purified fraction.

In this study of the C3-dependent ADP-ribosylation of rho in crude cytosol of bovine neutrophils and partially purified fractions, an unexpected finding was the increase in the amount of incorporated [^{32}P]ADP-ribose, expressed per milligram of protein, when the fractions tested were diluted. This increase was particularly striking on the total extent of ADP-ribosylation, but it was barely detected on the rate of ADP-ribosylation. Such a behavior could be explained by the release, upon dilution, of a loosely bound inhibitor which maskes the ADP-ribosylation site of rho. Some lipids have a similar effect. Most of them, at the concentrations found to be effective (millimolar), acted partly as detergents. A potential candidate for the bound inhibitory protein is GDI. However, GDI copurifies with rho until the last chromatographic step on Mono Q2, but at this step no further effect of dilution, detergents, or lipids is observed. A plausible explanation might be that the tightness of the association between rho and GDI loosens at the last step of the purification process. As an alternative, one may imagine that the masking of the C3 site on rho is due to a third protein that is loosely bound to the GDI–rho complex and that the unmasking to the C3 exoenzyme is due to the release of this protein.

In contrast to a number of lipids whose maximal effect on the ADP-ribosylation capacity of rho was obtained at millimolar concentrations, which makes any physiological role

of these compounds doubtful, PIP and PIP_2 were effective at micromolar concentrations, and therefore deserve some attention in terms of possible effects on molecular interactions of rho at the cell level. Evidence in favor of the control of the organization of the cytoskeleton by phosphoinositides has been recently discussed [cf. Stossel (1989) for a review]. Whether the enhancing effect of small concentrations of phosphoinositides on rho ADP-ribosylation reflects a specific role of these lipids in the functioning of rho, and consequently on the control of actin microfilament assembly by rho, remains to be demonstrated.

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