A rho Gene Product in Human Blood Platelets

I. IDENTIFICATION OF THE PLATELET SUBSTRATE FOR BOTULINUM C3 ADP-RIBOSYLTRANSFERASE AS rhoA PROTEIN*

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A substrate protein for botulinum C3 ADP-ribosyltransferase (C3 exoenzyme) in human platelets was purified to apparent homogeneity from the cytosol by ammonium sulfate fractionation and successive chromatography on columns of DEAE-Sepharose, hydroxylapatite, phenyl-Sepharose, and TSK phenyl-5PW. The purified protein yielded an amino acid sequence identical to that of rhoA protein. When platelet cytosol and membranes were incubated with C3 exoenzyme and $[^{32}P]NAD$ and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and isoelectric focusing, they gave only one $[^{32}P]$ADP-ribosylated band on each electrophoresis that showed an $M_r$ of 22,000 and a $pI$ of 6.0. The radioactive bands from the two fractions co-migrated with each other and with the $[^{32}P]$ADP-ribosylated purified protein. When these radioactive products were partially digested with either a-chymotrypsin or trypsin and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the same digestion pattern was found in the three samples. These results suggest that the ADP-ribosylation substrate for C3 exoenzyme in the platelet cytosol and membrane is rhoA protein and that it is the sole substrate detectable in human platelets.

ras-Related low molecular weight GTP-binding proteins constitute in mammalian cells a superfamily that now consists of more than 30 members, and accruing evidence suggests that these proteins play important roles in numerous biological processes such as cell growth, protein trafficking, secretion, and signal transduction (1, 2). Despite much investigation, however, biochemical pathways mediated by these proteins to elicit their functions still remain to be defined. C3 ADP-ribosyltransferase is an exoenzyme produced by certain strains of Clostridium botulinum (3–8). This enzyme specifically ADP-ribosylates several members of this superfamily. Currently, five proteins have been reported as substrates for the enzyme in mammalian tissues; they are three rho gene products (rho proteins) (rhoA, rhoB, and rhoC) and two rac proteins (rac1 and rac2) (9–14). C3 ADP-ribosyltransferase appears to be a useful tool to probe the function of these proteins because this enzyme modifies an asparagine residue in their putative effector domains and alters their functions (15). Investigations using this enzyme have suggested that these proteins are involved in the control of cytoskeletal organization. For example, Chardin et al. (13) showed that treatment of Vero cells with C3 ADP-ribosyltransferase results in modification of the substrates within the cells and, concomitantly, in disassembly of actin filaments and cell rounding. Paterson et al. (16) further demonstrated that microinjection of ADP-ribosylated rhoA protein into Swiss 3T3 cells induces similar dissolution of actin filaments and morphological changes, whereas the reappearance of actin filament networks is induced in contact-inhibited 3T3 cells by injection of Val14-rhoA mutant protein, which has a reduced GTPase activity and remains constitutively in the GTP-bound form. These results suggest that the ADP-riboylation substrate proteins, particularly rho proteins, have a role in the assembly of actin filament networks as well as in cell adhesion to the substratum.

The platelet is a cell fragment without a nucleus, the homogeneous preparation of which can easily be obtained. Much is known about its signal transduction mechanisms and morphological changes in response to extracellular stimuli. Upon receptor-mediated activation of phospholipase C, the platelet undergoes reorganization of cytoskeletal elements (i.e. actin filaments and microtubules) to cause striking shape changes and an adhesive property, which eventually lead to secretion and aggregation reactions (17). Thus, the platelet is an attractive system to study the stimulus-induced organization of actin networks; and the protein substrate ADP-ribosylated by botulinum C3 ADP-ribosyltransferase (C3 substrate), if present in the platelet, may play a role in these processes. This study was carried out taking these experimental advantages of platelets into account. We report in this paper the abundance, complexity, and identity of the C3 substrate in human platelets. The effects of C3 ADP-ribosyltransferase on platelet functions are reported in the accompanying paper (18).

EXPERIMENTAL PROCEDURES

Materials

C3 ADP-ribosyltransferase was purified from the culture filtrate of C. botulinum type C strain 003-S as described before (7, 8). $[^{32}P]NAD$ and $[^{35}S]GTPyS$ were obtained from Du Pont-New England Nuclear, DEAE-Sepharose CL-6B and phenyl-Sepharose CL-4B were obtained from Pharmacia LKB Biotechnology Inc., and hydroxyapatite was from Nakarai Tesque (Kyoto, Japan). A TSK phenyl-5PW column was purchased from Tosoh (Tokyo, Japan). Endoproteinase Asp-N and a-chymotrypsin were from Boehringer Mannheim and

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incubation for 3 h at 30 °C, the reaction was terminated by the addition of cold trichloroacetic acid. The precipitates were dissolved in 40 µl of Laemmli sample buffer (20) and subjected to 12.5% SDS-polyacrylamide gel electrophoresis. Guanine nucleotide binding was assayed as described previously (19).

**Platelet Preparations**

Platelets were prepared either from the peripheral blood of healthy human donors for subcellular distribution study or from outdated human platelet concentrates for protein purification. Platelets were freed of contaminating blood cells by centrifugation at 120 × g for 15 min and then pelleted by centrifugation at 2300 × g for 30 min. The platelet pellets were resuspended in 10 mM sodium phosphate, pH 7.0, 5 mM KCl, 135 mM NaCl, and 10 mM EDTA and washed three times by centrifugation. The washed pellets were suspended in 0.1 M of the original volume of 5 mM Tris-HCl, pH 7.4, 5 mM EGTA, 1 mM benzamidine hydrochloride, and 0.43 mM phenylmethylsulfonil fluoride and homogenized by 10 strokes in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 100,000 × g for 30 min for separation into the membrane and cytosol.

**Ammonium Sulfate Fractionation**—Ammonium sulfate was added to the cytosol fraction from outdated human platelets to 50% saturation. The mixture was stirred at 4 °C for 60 min and then centrifuged at 10,000 × g for 20 min. The supernatant was saved, and ammonium sulfate was added to 70% saturation. The mixture was stirred and centrifuged as described above. The precipitate was dissolved in 100 ml of TMDE buffer (20 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 1 mM diethiothreitol, and 1 mM EDTA) containing 1 µM GDP and 5 mM ethylene glycol and dialyzed against three changes of 5 liters of the same buffer.

**DEAE-Sepharose Column Chromatography**—The dialysate was applied to a column of DEAE-Sepharose CL-6B (2.5 cm, inner diameter, × 40 cm) equilibrated with TMDE buffer containing 5% ethylene glycol and 50 mM KCl. The column was washed with 600 ml of the equilibrium buffer. Elution was then performed with a linear gradient of 50–150 mM KCl (total volume of 1500 ml), and 10-ml fractions were collected. The substrate activity was eluted at 110 mM KCl in a single peak (Fig. 1). The peak fractions were pooled (DEAE fraction; 100 ml).

**Hydroxylapatite Column Chromatography**—The DEA fraction was applied to a column of hydroxylapatite (1.5 cm, inner diameter, × 25 cm) equilibrated with TMDE buffer containing 5% ethylene glycol and 0.1 M KCl. After the column was washed with 120 ml of the equilibrium buffer, elution was performed with a 400-ml linear gradient of 0–0.1 M potassium phosphate, pH 7.5. Fractions (7.5 ml) were collected. The

**Phenyl-Sepharose column chromatography of hydroxylapatite fraction.** Chromatography was performed as described in the text, and elution of the activities of the ADP-ribosylation substrate (•) and GTP·S binding (○) was determined. The straight line shows the linear gradients of the ammonium sulfate (30 to 50%) and ethylene glycol (5–75%) used for elution, and the dashed line indicates the elution profile of protein as determined by absorbance at 280 nm.

**Assays**

ADP-ribosylation was assayed essentially as described (19). In brief, a standard reaction mixture contained 100 mM Tris-HCl, pH 8.0, 10 mM thymidine, 10 mM nicotinamide, 10 mM dithiothreitol, 5 mM MgCl₂, 10 µM [³²P]NAD (2000 cpm/pmol), 0.1 µg of C3 ADP-ribosyltransferase, and the substrate in a total volume of 100 µl. After

**Purification of C3 Substrate**
major substrate activity was eluted at 40 mM potassium phosphate (Fig. 2). The active fractions were pooled (hydroxylapatite fraction; 82 ml).

Phenyl-Sepharose Column Chromatography—To the hydroxylapatite fraction was added ammonium sulfate to 30% saturation. The mixture was applied to a column of phenyl-Sepharose CL-4B (1.5 cm, inner diameter, × 5 cm) equilibrated with TMDE buffer containing 30% saturated ammonium sulfate, 5% ethylene glycol, and 1 μM GDP. The column was washed with 15 ml of the equilibrium buffer. Elution was performed with linear gradients of between 30 and 0% ammonium sulfate and 5 and 75% ethylene glycol (total volume of 100 ml), and 5-ml fractions were collected. The major substrate activity was eluted at ~8% ammonium sulfate, 66% ethylene glycol (Fig. 3). The fractions containing the major substrate activity were pooled (phenyl-Sepharose fraction; 30 ml).

TSK Phenyl-5PW Fast Protein Liquid Chromatography—The phenyl-Sepharose fraction was dialyzed against three changes of 3 liters of TMDE buffer containing 1 μM GDP and 5% ethylene glycol. To the dialysate was added ammonium sulfate to 30% saturation. The mixture was applied to a TSK phenyl-5PW column (7.5 mm, inner diameter,× 7.5 cm) equilibrated with TMDE buffer containing 30% saturated ammonium sulfate, 5% ethylene glycol, and 1 μM GDP. The column was washed with 12 ml of the TMDE buffer containing 15% saturated ammonium sulfate, 40% ethylene glycol, and 1 μM GDP. Elution was performed with linear gradients of between 15 and 0% ammonium sulfate and 40 and 75% ethylene glycol at a flow rate of 0.2 ml/min, and 0.8-ml fractions were collected. The substrate activity was eluted at 5% ammonium sulfate, 63% ethylene glycol in a single peak (Fig. 4). The active fractions were pooled and used as the final preparation.

Amino Acid Sequence Analysis

The purified sample containing 25 μg of protein was dialyzed against three changes of 1 liter of 50 mM sodium phosphate, pH 8.0, and 0.01% SDS. To the dialysate was added 0.5 μg of endoproteinase Asp-N, and the mixture was incubated at 37 °C for 12 h. Proteolytic peptides were isolated by reversed-phase HPLC using an Aquapore RP-300 column (2.1 mm, inner diameter, × 30 mm) on a Beckman HPLC system Gold (Fig. 5), and the isolated peptides were subjected to amino acid sequencing by automated Edman degradation with an Applied Biosystems Model 477A Gas-Phase Sequencer equipped with a Model 120A phenylthiohydantoin-derivative analyzer as described previously (8).

Miscellaneous Procedures

SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (20). Isoelectric focusing was carried out by the method of O'Farrell (21) using a Pharmacia LKB Biotechnology 2217 Multiphor system as described previously (8).

RESULTS

Purification of Substrate for C3 ADP-riboseyltransferase in Human Platelets and Its Identification as rhoA Protein—

Table I shows the subcellular distribution of the C3 substrate activity in human platelets. Platelets contain >20 pmol of C3 substrate/mg of protein. Comparison of this value with our previous study (19) on the tissue distribution of the C3 substrate reveals that the platelet is as rich in the C3 substrate as the brain, spinal cord, and adrenal gland. Although the substrate activity is found both in the cytosol and membrane fractions, the cytosol contains the larger amount and the higher concentration of the substrate activity. We therefore used the cytosol as a starting material for the purification and characterization of the C3 substrate.

The C3 substrate in human platelet cytosol was purified by ammonium sulfate fractionation and successive chromatography on columns of DEAE-Sepharose, hydroxylapatite, phenyl-Sepharose, and TSK phenyl-5PW as described under "Experimental Procedures." A summary of this purification
**TABLE II**

| Step                  | Protein | ADP-ribosylation substrate |
|-----------------------|---------|----------------------------|
|                       | mg      | nmol | nmol/mg | -fold | %   |
| Cytosol               | 6422    | 178.6| 0.028   | 1     | 100 |
| Ammonium sulfate      | 1240    | 81.0 | 0.065   | 2     | 45  |
| DEAE-Sepharose        | 34.3    | 22.7 | 0.66    | 24    | 13  |
| Hydroxylapatite       | 7.1     | 10.1 | 1.42    | 51    | 5.6 |
| Phenyl-Sepharose      | 1.0     | 4.8  | 4.80    | 173   | 2.7 |
| Phenyl-5PW            | 0.032   | 1.02 | 31.80   | 1144  | 0.5 |

**FIG. 6.** Comparison of partial amino acid sequences of purified substrate with amino acid sequences deduced from human rho and rac cDNAs. The amino acid sequences of peptide peaks 1-6 shown in Fig. 5 were determined as described under "Experimental Procedures." The deduced amino acid sequences of human rho and rac cDNAs were taken from published data (14, 23, 24). Partial amino acid sequences are shown (shaded) between those of rhoA and rhoB proteins and correspond (from left to right) to peptide peaks 5, 2, 4, 1, 5, and 6, respectively.

is shown in Table II. Approximately 1100-fold purification was achieved with a yield of 0.5%. Only a single peak of protein with GTP$\gamma$S binding activity was purified as the ADP-ribosylation substrate. The final preparation gave a single protein band on SDS-polyacrylamide gel electrophoresis at an $M_r$ of 22,000 (Fig. 4). The purified protein incorporated $\sim$0.7 mol of ADP-ribose/mol in the C3 enzyme-catalyzed reaction and bound GTP$\gamma$S maximally to 0.8 mol/mol with a $K_d$ of 50 nM (data not shown).

To identify the C3 substrate purified as described above, we digested the purified protein with endoproteinase Asp-N, and the generated peptides were separated by reversed-phase HPLC and sequenced. As shown in Fig. 6, all the sequences obtained were found in the deduced amino acid sequence of human rhoA (23), but not in those of any other substrate proteins for C3 ADP-ribosyltransferase (14, 24). These results clearly identify the purified substrate protein as rhoA protein.

**DISCUSSION**

In this study, we purified the C3 substrate from human platelet cytosol and identified it as rhoA protein. To our knowledge, this is the first report showing the presence of rhoA protein in the platelet. Other low molecular weight GTP-

other than the one we purified in this study, we [$^{32}$P]ADP-ribosylated substrate(s) in the crude cytosol and membranes and compared them with the purified protein on SDS-polyacrylamide gel electrophoresis and isoelectric focusing. As shown in Fig. 7, the substrate from both fractions yielded a single [$^{32}$P]ADP-ribosylated protein band that co-migrated with that of purified rhoA protein. No other bands were discernible even after prolonged exposure or on isoelectric focusing between pH 3.5 and 10 (data not shown).

The identities of these [$^{32}$P]ADP-ribosylated products were further studied by limited protease digestion (Fig. 8). When digested with $\alpha$-chymotrypsin, both the membrane and cytosolic C3 substrates were converted to 10- and 8-kDa fragments via 14- and 12-kDa intermediates, which further degraded into smaller peptides. Digestion of [$^{32}$P]ADP-ribosylated rhoA protein yielded an indistinguishable pattern. The patterns obtained by limited digestion with trypsin were also indistinguishable among the three samples (data not shown). These results, together with the findings that rho proteins are found in both the membrane and soluble fractions in many types of cells (25, 26), strongly suggest that rhoA protein is the only C3 substrate in human platelets.
binding proteins found in platelets by previous studies are rap 1A and B, c25KG, rac p21, ral p23, and G25K (CDC42HS) (1, 27-30). We then examined the presence of C3 substrates other than rhoA protein in the platelet by SDS-polyacrylamide gel electrophoresis and autoradiography. Samples taken at the same time also contained purified rhoA protein, respectively. The cytosol containing purified rhoA protein was subjected to 20% SDS-polyacrylamide gel electrophoresis and autoradiography. Samples taken at the same time are shown in three and are (from left to right) the cytosol, membranes, and purified rhoA protein, respectively.

Thus, our results strongly suggest that rhoA protein is by far the predominant, if not sole, C3 substrate in human platelet and that the platelet may be a good model system to study the function of this protein. Multiple species of C3 substrates are usually present in other types of cells, and most of them are not identified, making it difficult to assign the observed biological effects of C3 ADP-ribosyltransferase to any member of rho proteins. Because we now identified rhoA protein as the sole target for C3 ADP-ribosyltransferase in human platelets, the effect of the ADP-ribosylation on platelets can be reasonably attributed to the function of rhoA protein. The high level of rhoA protein in platelets suggests that components that associate with rhoA protein are more abundant in platelets than in other cells. Indeed, we detected the highest level of the rho-specific GTPase-activating protein activity in platelets. It was found very recently that G_P, a low molecular weight GTP-binding protein purified from human placenta and platelets (32, 33), is a human homolog of yeast cell division-cycle proteins CDC42 (34) (CDC42HS) (35, 36) and that its GTPase-activating protein purified from human platelets also stimulates the GTPase activity of recombinant rhoA protein (37). This, together with our present finding, raises an intriguing possibility that the biochemical pathways mediated by G_P and rhoA protein are interrelated or “cross-talking” in the platelet. Further study on the function of rhoA protein in the platelet would shed light on these interactions.

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