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Production of monoclonal antibodies that inhibit ADP-ribosylation of small GTP-binding proteins catalyzed by *Clostridium botulinum* ADP-ribosyltransferase C3

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Four monoclonal antibodies that inhibited ADP-ribosylation of 23 kDa protein(s) of ascidian eggs catalyzed by *Clostridium botulinum* ADP-ribosyltransferase C3 were produced. They also inhibited C3-catalyzed ADP-ribosylation of the 24 kDa protein of rat liver cytosol. By the immunoprecipitation technique, it was found that they recognized small GTP-binding proteins of ascidian eggs and mammalian brains, but did not interact with the rat brain activator of the ADP-ribosyltransferase reaction. The antibody can also immunoprecipitate recombinant Rho A irrespective as to whether the Rho A is the GDP-bound form or the GTPrS-bound form. Thus the antibodies are novel and useful tools in analyzing the physiological roles of the Rho family of GTP-binding proteins.

ADP-ribosylation; GTP-binding protein; Monoclonal antibody; *Clostridium botulinum*; C3

1. INTRODUCTION

Rho and related proteins, members of the Ras superfamily of GTP-binding proteins [1,2], are specifically ADP-ribosylated by botulinum exoenzyme C3 produced by *Clostridium botulinum* type C or D [3,4] and also by the epidermal cell differentiation inhibitor (EDIN) produced by *Staphylococcus aureus* [5]. Physiological roles of Rho have been investigated by microinjection into several cells or by treatment of the cells using botulinum exoenzyme C3 [6–9], constitutively active or dominant negative recombinants of Rho [10–12] and an inhibitory GDP/GTP exchange protein of Rho (rho GDI) [9,13,14]. Results suggest that Rho regulates actin stress fiber formation. With respect to the roles of Rac, a Rho-related protein, it is proposed that Rac regulates actin membrane ruffling [15] and also plays a role in the activation complex of NADPH oxidase [16].

On the other hand, one of the authors (T.K.) [17] reported the presence of the endogenous botulinum C3-like enzyme that catalyzes ADP-ribosylation of small GTP-binding proteins, which suggests the possibility that the functions of the small GTP-binding proteins might be regulated through endogenous ADP-ribosylation although little is known about the mechanisms under physiological circumstances.

In this study, we report on the production of monoclonal antibodies that inhibit ADP-ribosylation of small GTP-binding proteins catalyzed by botulinum exoenzyme C3 and present evidence that the antibodies recognize the small GTP-binding proteins. To our knowledge, this is the first report on such specific monoclonal antibodies directed against the small GTP-binding proteins. The antibodies are expected to be useful tools to analyze the physiological roles of the Rho family of GTP-binding proteins.

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2. MATERIALS AND METHODS

2.1 *Materials*

Unfertilized eggs of the ascidian, *Halocynthia roretzi*, were collected as described previously [18]. Botulinum exoenzyme C3 was purified from the culture supernatant of *C botulinum* strain D-1873 as described previously [19]. Protein G-agarose was purchased from Sigma (St. Louis, MO, USA). Anti-mouse IgG was from Vector Laboratories, Inc. (Burlingame, CA, USA). [^{32}P]NAD (800 Ci/mmol) was from New England Nuclear.

2.2. *Immunization and preparation of hybridoma cells*

Egg membrane preparations and a partially-purified preparation of small GTP-binding protein, both of which were used for immunization, were prepared as follows: ascidian unfertilized eggs were homogenized in 20 mM Tris-HCl (pH 8.0), containing 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM EDTA, 0.001 mM GDP, 0.01 mM diisopropylfluorophosphate and 0.1 mM leupeptin. The homogenate was centrifuged at 800 × *g* for 10 min and at 10,000 × *g* for 30 min and the resulting precipitate was used as the membrane preparation. The membrane preparation was then solubilized with 1% CHAPS and the supernatant obtained after centrifugation (100,000 × *g*, 1 h) was referred to as the CHAPS extract. The extract was applied to a QAE-Sephadex A-50 (Pharmacia) column previously equilibrated with 20 mM Tris-HCl (pH 7.5) containing 5 mM $MgCl_2$, 1 mM DTT, 1 mM

EDTA and 0.005% Lubrol PX. The column was washed with the equilibration buffer and eluted by increasing the NaCl concentration to 0.3 M. The NaCl-eluted fraction was used as a preparation of small GTP-binding protein.

The egg membrane preparation was emulsified with an equal volume of Freund's complete adjuvant and injected intraperitoneally into female Balb/c mice (0.2 mg membrane protein/animal). An initial injection was followed by two intraperitoneal boosters of partially purified small GTP-binding protein (0.2 mg of protein) emulsified with an equal volume of Freund's incomplete adjuvant at 2-week intervals. On the third day after the final immunization, the spleen cells were harvested and fused with myeloma cells (strain P3X63-Ag8.653) by standard polyethylene glycol method [20]. Hybridoma cells thus obtained were cultured at 37°C under 7% CO_2 in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo) containing 20% fetal calf serum (Whittaker Bioproducts, Inc., Walkersville, MA, USA).

2.3. *Screening of hybridoma cells*

Antibody production was checked on the basis of inhibition of ADP-ribosylation of the ascidian egg small GTP-binding protein catalyzed by botulinum exoenzyme C3. Culture medium of hybridoma cells was mixed with an equal volume of the CHAPS extract of ascidian eggs, incubated at 20°C for 1 h, and then added to the reaction mixture consisting of 20 mM Tris-HCl (pH 8.0), 10 mM thymidine, 1 mM EDTA, 1 mM DTT, 1.5 mM $MgCl_2$, 0.001 mM [^{32}P]NAD and 0.001 mg/ml exoenzyme C3 in a final volume of 0.04 ml. After incubation at 25°C for 1 h, the reaction was terminated by the addition of 0.1 ml of 0.1 M Tris-HCl (pH 8.0) containing 0.5% SDS and 5 mg/ml BSA, followed by 1 ml of 5% trichloroacetic acid (TCA). Precipitates were filtered through a GF/C filter (Whatman) and the filter was extensively washed with 5% TCA. Radioactivity remaining on the filter was measured by liquid scintillation counting. Hybridoma cells, culture media of which inhibited the incorporation of radioactivity into the TCA-insoluble fraction, were referred to as positive ones, and subjected to limiting dilution for cloning of positive cell lines. Four antibody-producing clones (A5, C4, C7 and G5) were established.

2.4. *Typification and quantification of monoclonal antibodies*

Subclasses of monoclonal antibodies were determined using the mouse monoclonal antibody isotyping kit (Amersham) according to manufacturer's protocol.

Amounts of monoclonal antibodies contained in the culture media were measured by a sandwich enzyme-linked immunosorbent assay (ELISA) as follows; purified immunoglobulin G (IgG) against mouse IgG (anti-mouse IgG, Vector) (0.01 mg/ml) was adsorbed to a 96-well ELISA plate after standing at 4°C overnight. After washing and blocking, culture medium was added to the well and the plate was kept standing at room temperature for 2 h. After washing, biotin-conjugated anti-mouse IgG (Vector) and then avidin–biotinylated peroxidase complex were added to the well and incubated at room temperature for 2 h and for 30 min, respectively. The color was developed using 0.02% H_2O_2 and 0.2 mg/ml 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) as substrates.

2.5. *SDS-polyacrylamide gel electrophoresis*

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli [21] for determination of the molecular weight of the C3 substrate that was ADP-ribosylated by exoenzyme C3. Gels were stained with Coomassie brilliant blue and subjected to autoradiography.

2.6. *Preparation of the small GTP-binding protein and the activator of the ADP-ribosyltransferase reaction*

Preparation of small GTP-binding proteins (C3 substrates) from ascidian eggs is described in detail elsewhere. Briefly, the CHAPS extract was successively subjected to four chromatographies on columns of DEAE-Toyopearl (Tosoh), phenyl-Sepharose CL-4B (Pharmacia), hydroxylapatite (Pentax) (HPLC), and Mono Q (Pharmacia) (FPLC). Small GTP-binding proteins were monitored by C3-catalyzed ADP-ribosylation. The ADP-ribosyltransferase reaction of the final purified preparation obtained from the Mono Q column did not require the presence of any activator.

Small GTP-binding proteins (C3 substrates) and activator(s) of the ADP-ribosyltransferase reaction were prepared from rat brain as described previously [22]. Rat brain was homogenized in 10 mM Tris-HCl (pH 7.5) containing 10% sucrose and 0.01 mM leupeptin and the homogenate was centrifuged at $10,000 \times g$ for 30 min and at $100,000 \times g$ for 60 min. The resulting supernatant (cytosol) was subjected to a DEAE-Toyopearl 650M column previously equilibrated with 10 mM Tris-HCl (pH 7.5) containing 10% sucrose, 0.01 mM leupeptin, 1 mM $MgCl_2$ and 20 mM NaCl. After washing, the column was eluted with a 20–300 mM NaCl linear gradient in equilibration buffer. The GTP-binding protein was eluted at 50 mM NaCl, while the activator eluted around 200 mM NaCl. Both preparations were used for immunoprecipitation experiments.

Rat liver cytosol was prepared according to the method used for the preparation of rat brain cytosol as described above. Purified C3 substrate GTP-binding protein was prepared from bovine brain as described previously [23]. Recombinant Rho A was purified from the soluble fraction of *Escherichia coli* in which Rho A was overexpressed using a plasmid, pGEX-2T *rho* A [24]. The purified C3 substrate and recombinant Rho A proteins were purified as GDP-bound forms. Where indicated, GTPrS-bound form of the recombinant Rho A was prepared by incubation with 0.02 mM GTPrS at 30°C for 30 min in a reaction mixture containing 0.003 mM $MgCl_2$ [23]. The plasmid and cDNA of *rho* A were kindly provided by Drs. Y. Takai (Kobe University School of Medicine, Japan) and P. Madaule (Centre National de la Recherche Scientifique Laboratoire, Gif sur Yvette, France).

2.7. *Immunoprecipitation*

Anti-mouse IgG was diluted to 0.1 mg/ml with 20 mM Tris-HCl (pH 8.5) containing 0.15 M NaCl, mixed with protein G-agarose (the swollen volume was approximately 0.05 ml) and incubated at room temperature for 4 h. Monoclonal antibody solution (0.005 mg/ml) was added to the gel recovered by centrifugation and incubated at room temperature for 4 h. The gel was washed with the buffer by centrifugation and used as a complex consisting of monoclonal antibody, anti-mouse IgG and protein G-agarose.

The gel was added to either the small GTP-binding protein (C3 substrate) preparation or to the activator preparation and the mixture was incubated at 4°C overnight. After the gel was removed by centrifugation, the resulting supernatant was recovered. The remaining amounts in the supernatants of the GTP-binding protein and the activator were determined by C3-catalyzed ADP-ribosylation after the addition of a sufficient amount of the activator and a defined amount of the GTP-binding protein, respectively.

3. RESULTS AND DISCUSSION

By screening on the basis of inhibition of ADP-ribosylation of small GTP-binding proteins catalyzed by botulinum exoenzyme C3, we succeeded in establishing four hybridoma cell clones producing monoclonal antibodies that inhibit C3-catalyzed ADP-ribosylation.

Table I

Established hybridoma clones producing monoclonal antibodies that inhibit C3-catalyzed ADP-ribosylation of small GTP-binding proteins

Clone	Subclass	Light chain
A5	IgG3	lambda
C4	IgG2b	kappa
C7	IgG3	kappa
G5	IgG2b	kappa

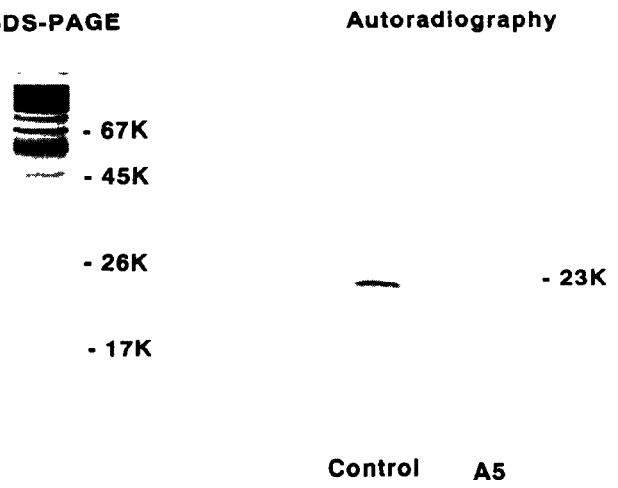

Fig. 1. Inhibition by monoclonal antibody A5 of C3-catalyzed ADP-ribosylation of 23 kDa protein of ascidian egg membrane. The CHAPS extract was previously incubated with A5 antibody or control IgG, and the ADP-ribosylation reaction was carried out. The reaction mixture was then subjected to SDS-PAGE, followed by autoradiography.

Four monoclonal antibodies were classified as IgG (Table I). An autoradiogram (Fig. 1) shows that the botulinum exoenzyme C3 catalyzed ADP-ribosylation of small GTP-binding protein(s) of ascidian eggs has a molecular mass of 23 kDa and that the monoclonal antibody (A5) inhibited ADP-ribosylation.

Fig. 2 shows dependency on the antibody concentration of its inhibition of C3-catalyzed ADP-ribosylation of GTP-binding proteins of ascidian egg membrane and rat liver cytosol (in the latter case, exoenzyme C3 ADP-ribosylated a 24 kDa protein). Any antibody inhibited the ADP-ribosylation of the two GTP-binding proteins in a similar concentration-dependent manner. When recombinant Rho A was used as a C3 substrate, the same results were obtained (data not shown).

To define antigen molecule(s) that are recognized by the monoclonal antibodies thus prepared, we used them for Western blot analysis by the method of Towbin et al. [25]. But, however, no detectable signs were observed, indicating that the antibodies are unable to react with the antigen(s) previously treated with 1% SDS. Thus the antibodies may recognize the gross structure of the antigen.

One of the authors [22] previously reported the presence in bovine brain of activator(s) that activate ADP-ribosyltransferase reaction catalyzed by exoenzyme C3. Since we used the CHAPS extract of ascidian eggs as a C3 substrate for the ADP-ribosyltransferase reaction during screening of hybridoma cells, two possibilities of the mechanisms through which the antibodies inhibit C3-catalyzed ADP-ribosylation of small GTP-binding proteins can be considered. One is the possibility that the antibody inhibits the ADP-ribosyltransferase reaction through its binding to an ADP-ribosylation site on

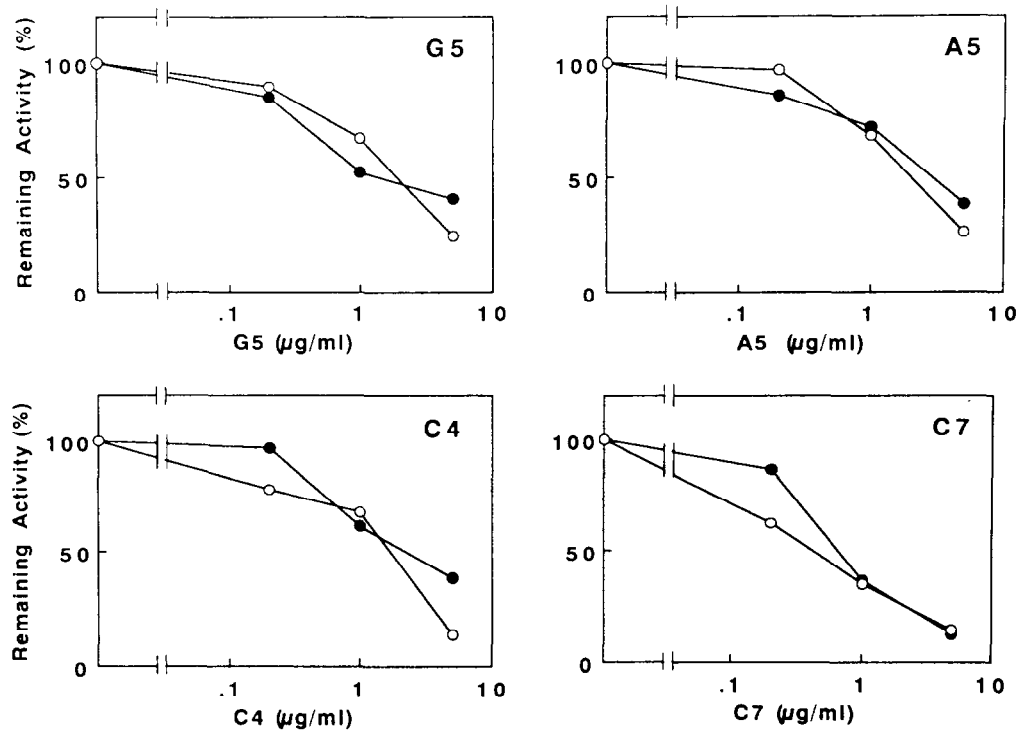

Fig. 2. Inhibition by monoclonal antibodies G5, A5, C4 and C7 of C3-catalyzed ADP-ribosylation using ascidian egg CHAPS extract (○) and rat liver cytosol (●) as C3 substrates. The CHAPS extract or the cytosol was previously incubated with each monoclonal antibody and subjected to the ADP-ribosylation reaction. After termination by the addition of 0.5% SDS and then 5% TCA, the precipitates were filtered through a GF/C filter. Radioactivity remaining on the filter was measured. The value in the absence of antibody was defined as 100%.

Table II

Immunoprecipitation with monoclonal antibodies

Preparation	Amount in the supernatant (%)[a]				
	Control[b]	A5	C4	C7	G5
(a) Ascidian egg preparation					
1. Small GTP-binding protein	100	32	47	35	53
(b) Rat brain preparation					
1. Small GTP-binding protein	100	38	64	59	72
2. Activator	100	95	97	96	98
(c) C3 substrate GTP-binding Protein from bovine brain	100	45	70	48	74
(d) Recombinant Rho A					
1. GDP-bound form	100	33	–	–	–
2. GTPrS-bound form	100	56	–	–	–

Each preparation was immunoprecipitated with either of monoclonal antibodies and the amount remaining in the supernatant was measured as described in section 2.

[a] The amount in the presence of control IgG was defined as 100%.

[b] Control mouse IgG.

the small GTP-binding protein, while the other is that it inhibits activation of the reaction through its binding to the activator. To clarify this point, we investigated which of the GTP-binding proteins or the activators can be immunoprecipitated by the antibody (Table II). In the case of ascidian eggs, we did not succeed in separating the GTP-binding protein from the activator although high purification of the former was achieved. However, an immunoprecipitation experiment with ascidian egg GTP-binding protein, ADP-ribosylation of which is not required for the presence of the activator, shows that four monoclonal antibodies can immunoprecipitate the GTP-binding protein (Table IIa).

Next, we tried to isolate the small GTP-binding protein(s) and the activator from rat brain cytosol. The DEAE-Toyopearl chromatography gave a good separation between them. When the former was immunoprecipitated with the antibody, the remaining amount of the former in the supernatant was decreased (Table IIb). On the other hand, the remaining amount of the latter remained unchanged after immunoprecipitation. Thus the four monoclonal antibodies can recognize the small GTP-binding protein, but did not interact with the activator.

Finally, we confirmed the immunoreactivity of the monoclonal antibody with the small GTP-binding protein using pure C3 substrate GTP-binding protein of bovine brain (Table IIc) and recombinant Rho A (Table IId). In fact, four monoclonal antibodies can immunoprecipitate the pure C3 substrate GTP-binding protein. Furthermore, the antibody A5 can immunoprecipitate either the GDP-bound form or GTPrS-bound form of the recombinant Rho A, although it seems that the antibody can interact with the former form a little stronger than the latter. In conclusion, the monoclonal antibodies prepared in this study can recognize epitopes of the Rho family of GTP-binding proteins, common from the protochordates to the mammals.

It has been reported that Asn^{41} of Rho is ADP-ribosylated by exoenzyme C3 [26]. Since this site is thought to be located in the putative effector domain of Rho, it is likely that the monoclonal antibody prepared in this study can block the interaction between Rho and its regulatory proteins such as rho GAP. Alternatively, it may inhibit putative endogenous ADP-ribosylation of Rho. In our preliminary experiments, microinjection of this monoclonal antibody into ascidian egg resulted in the inhibition of egg activation and fertilization, which suggests the involvement of Rho or Rho-related proteins in these phenomena. Thus the antibodies are novel and useful tools to analyze the functions of Rho and related proteins.

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