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Purification of GTP-binding proteins from bovine brain membranes

Identification of heterogeneity of the α-subunit of G_o proteins

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Using high-resolution Mono Q column chromatography, we purified 6 distinct peaks of GTP-binding proteins from bovine brain membranes. Five of them consisted of 3 polypeptides with αβγ-subunits and served as the substrate of islet-activating protein (IAP), pertussis toxin. The other one was purified as α-subunit alone and was also ADP-ribosylated by IAP in the presence of βγ-subunits. When each α-subunit was characterized by immunoblot analysis using various antibodies with defined specificity, the two of them were identified as G_{i-1} and G_{i-2}, and other 4 appeared to be G_o or G_o-like G proteins. The α-subunits of immunologically G_o-like proteins were apparently distinguishable from one another on elution profiles from the Mono Q column. Thus, there was a heterogeneity of the α-subunit of G_o in the brain membranes.

ADP-ribosylation; GTP-binding protein; Islet-activating protein

1. INTRODUCTION

In a variety of vertebrate cells, GTP-binding proteins (G-proteins) function as transducers that carry signals from activated receptors to effectors such as enzymes or ion channels [1]. G-proteins have been purified from several sources as heterotrimers with an αβγ-subunit structure. Their α-subunits which bind GTP (or GDP) and are substrates for ADP-ribosylation catalyzed by bacterial toxins such as islet-activating protein (IAP), pertussis toxin. Molecular cloning of the α-subunit genes [2] and cDNAs [3-5] has indicated the existence of at least 4 genes for the α of G_{i-1}, G_{i-2}, G_{i-3} and G_o serving as the substrate of IAP besides two α-subunits of transducin (G_{t-1} and G_{t-2}). Among them, 3 IAP-substrate G-proteins (G_{i-1}, G_{i-2} and G_o) have recently been purified from brain membranes [6,7] and compared with predicted amino acid sequences from the α genes and cDNAs [8]. More recently, Goldsmith et al. have purified a G_o-like protein, in addition to the above 3 G-proteins, from bovine brain membranes [9]. Thus, there are multiple homologous, but distinct, IAP-sensitive G-proteins in brain tissues. Here, we report the purification of at least 5 αβγ-heterotrimeric G-proteins serving as the substrate of IAP and a free α-subunit from bovine brain. These purified G-proteins are characterized and identified using antibodies with defined specificity, in addition to the elution profiles from a high-resolution anion-exchange column chromatography.

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2. MATERIALS AND METHODS

2.1. *Materials*

^{35}S-labeled guanosine 5'-*O*-(thio)triphosphate, GTPγS, (48.5 TBq/mmol), ^{35}P-labeled NAD (29.6 TBq/mmol) and ^{125}I-labeled goat anti-rabbit IgG F(ab')^2 (74-370 MBq/μg) were purchased from Dupont New England Nuclear. The sources of all other reagents used are those described in [6,10-12].

2.2. *Purification of G-proteins from bovine brain*

Bovine brains were washed and homogenized with a buffer consisting of 10 mM Tris-HCl (pH 7.5), 10% (w/v) sucrose and 25 kallikrein inhibitory units (KIU) per ml of aprotinin (referred henceforth to as TSA). The homogenate was centrifuged at 10000 rpm for 30 min in a Beckman JA-10 rotor, and the precipitate, after being washed again with TSA, was homogenized with 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol (DTT), 25 KIU/ml of aprotinin (TEDA) containing 100 mM NaCl. After centrifugation at 14000 rpm for 30 min in a Beckman JA-14 rotor, the precipitate was resuspended with an equal volume of TEDA and stored as the membrane preparation of bovine brains at −80°C.

The membranes (350 g) were extracted at 0-4°C for 1 h with 700 ml of TEDA containing 1.5% (w/v) Na cholate and 12.5 mM NaCl and centrifuged at 40000 rpm for 90 min in a Beckman 45-Ti rotor. The clear supernatant (approximately 900 ml) was applied to a column (5 × 25 cm) of DEAE-Sephacel (Pharmacia LKB Biotechnology Inc.) which had been equilibrated with 25 mM NaCl in TEDA/1% Na cholate and then eluted with a linear gradient of NaCl (25-250 mM; 1.4 liters) in the same buffer. The major fractions containing GTPγS-binding activity were concentrated to 20 ml using an ultrafiltration

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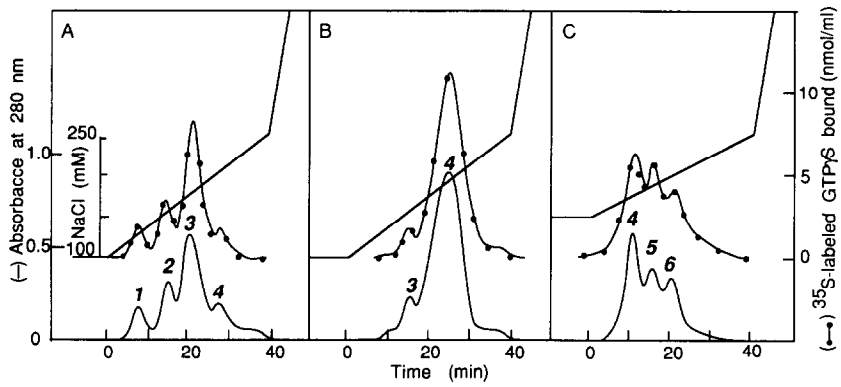

Fig.1. Elution profiles of GTPγS-binding activity from Mono Q column chromatography. Each of 3 major G-protein-rich fractions containing G_{i-1}, G_o and G_{i-2} [6,9] were separately applied to Mono Q column and eluted as described in section 2.2, and the elution profiles of GTPγS-binding activity are illustrated in panels (A), (B) and (C), respectively. Aliquots (5 μl) of fractions were assayed for GTPγS-binding activity (●——●). The absorbance at 280 nm of the eluted protein was also monitored (——).

system of NM-3 (Asahikasei), and the concentrate was fractionated on a gel filtration column (4 × 90 cm) of Sephacryl S-300 (HR) (Pharmacia-LKB) which had been equilibrated with 100 mM NaCl and 1 μM GDP in the TEDA buffer. The major fractions containing the activities of GTPγS binding and the substrate for IAP-catalyzed ADP-ribosylation were pooled and diluted with 3 vols of TED (containing no aprotinin) fortified with 300 mM NaCl and 1 μM GDP. The diluted sample was applied to a column (2 × 16 cm) of phenyl-Sepharose CL-4B (Pharmacia-LKB) which had been equilibrated with 250 mM NaCl, 0.25% Na cholate and 1 μM GDP in TED and eluted with a linear gradient (200 ml) starting with 0.25% Na cholate and 250 mM NaCl and ending with 1% Na cholate and 0 mM NaCl in the TED buffer. The major fractions containing the IAP-substrate activity were pooled and, after being concentrated to about 5 ml using the NM-3, diluted with 3 vols of 1% (w/v) Lubrol-PX in TED. The diluted sample was applied to a column (1 × 8 cm) of DEAE-Toyopearl 650 (S) (Tosoh) which had been equilibrated with 50 mM NaCl in TED/0.75% Lubrol-PX and eluted with a linear gradient of NaCl (50–200 mM; 60 ml) in the same buffer. This chromatography resulted in a partial separation of 3 peaks of IAP-substrate and GTP-binding activities; the first (I), second (II) and third (III) peaks mainly contained G_{i-1}, G_o and G_{i-2}, respectively, as reported previously [6,9].

Each of the 3 peaks (I–III), after being concentrated by a column of hydroxyapatite (HCA-100S; Mitsui Toatsu Chemicals), was further applied to a Mono Q HR5/5 column (Pharmacia-LKB) which had been equilibrated with 100 mM NaCl in TED/0.7% (w/v) 3[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Chaps) and then eluted at a flow rate of 0.75 ml/min with the following series of NaCl gradients using a Pharmacia FPLC system; 100 mM for 2 min or 150 mM for 2 min; 100–250 mM over 40 min or 150–250 mM over 40 min; 250–1000 mM over 5 min. The panels A, B and C in fig.1 detail the activities measured in the fractions eluted from the Mono Q columns to which the peaks I, II and III from the DEAE Toyopearl had been applied, respectively. Thus, 6 peaks of G-proteins serving as the substrate for IAP were purified to almost homogeneity (see fig.1A,B and C). These G-proteins were concentrated using an ultrafiltration system of UFPI-LGC (Millipore) and filtered through a column of Sephadex G50 (fine; Pharmacia-LKB) in 50 mM sodium *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.4) and 0.7% Chaps prior to storage at −80°C.

2.3. *Assay of activities*

G-proteins serving as the substrate for IAP were identified by their abilities to be ^{32}P-labeled, ADP-ribosylated in the presence of IAP and ^{32}P-labeled NAD, and to bind ^{35}S-labeled GTPγS as in [10]. Electrophoresis of polypeptides through SDS-polyacrylamide gels and autoradiography are described in [10]. The samples were alkylated with *N*-ethylmaleimide prior to electrophoresis as in [10]. Protein was quantitated by staining with Amido black with bovine serum albumin as a standard protein [13].

2.4. *Immunoblot procedure*

Immunoblot analysis was performed essentially as in [6,11,13]. Antibodies used were: affinity-purified rabbit polyclonal antibodies prepared against the α of G_o purified from rat brain which specifically reacted with the α_o (see [6,11]); antisera raised against synthetic decapeptides corresponding to the divergent sequences of three cDNAs encoding the α of G_{i-1}, G_{i-2} and G_{i-3} [13], which were generous gifts from Dr Y. Kanaho, Smith Kline and French Laboratories.

3. RESULTS AND DISCUSSION

In the previous paper [6], we have purified 3 major peaks of G-proteins serving as the substrate of IAP from brain membranes. They predominantly contained G_{i-1}, G_o and G_{i-2}, respectively [8], which were eluted from an anion-exchange column with a linear NaCl gradient. During the course of our purification studies of G-proteins, however, we found that there were many significant peaks of G-proteins other than the previously identified G-proteins. The panels A, B and C in fig.1 show elution profiles of GTPγS-binding activity from high-resolution Mono Q anion-exchange columns to which the G_{i-1}, G_o and G_{i-2}-rich fractions were applied and eluted from with a NaCl gradient, respectively. Each of the Mono Q chromatographies resulted in a separation of more than two peaks of GTPγS-binding activity. For simplicity, these peaks are severally henceforth referred to as peak 1–6, in order of elution from the column; peaks 1, 2, 3, 4, 5 and 6 were eluted at about 145, 165, 175, 195, 205 and 215 mM NaCl, respectively, under the present conditions.

Each of the 6 peaks was analyzed by SDS-PAGE and the protein stain of the gel is illustrated in fig.2A. Peaks 2–6 consisted of 3 polypeptides with 39–41-kDa α- and βγ-subunits (the γ-subunits had not appeared on the gel), while peak 1 contained only the 39-kDa α-subunit. The α-subunits of peaks 2–6 were ^{32}P-labeled, ADP-

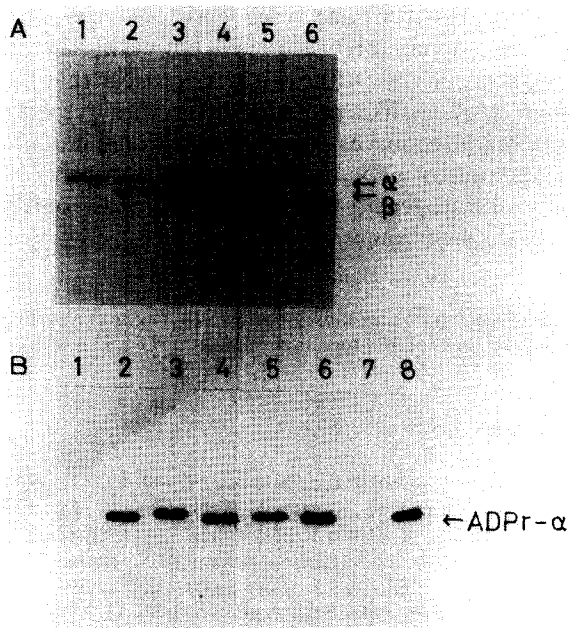

Fig.2. SDS-PAGE and IAP-catalyzed ADP-ribosylation of purified G-proteins. (A) G-proteins (0.2 μg) purified by the Mono Q column were subjected to SDS-polyacrylamide gel (12%) electrophoresis and stained with Coomassie blue. γ-Subunits did not appear on the gel. (B) G-proteins which had been ^{32}P-labeled, ADP-ribosylated by IAP in the presence of ^{32}P-labeled NAD (and purified $\beta\gamma$-subunits for lane 8) were subjected to autoradiography as described in section 2.3. Lanes 1–6, peaks 1–6 shown in fig.1, respectively; 7, peak 1; 8, peak 1 plus $\beta\gamma$-subunits.

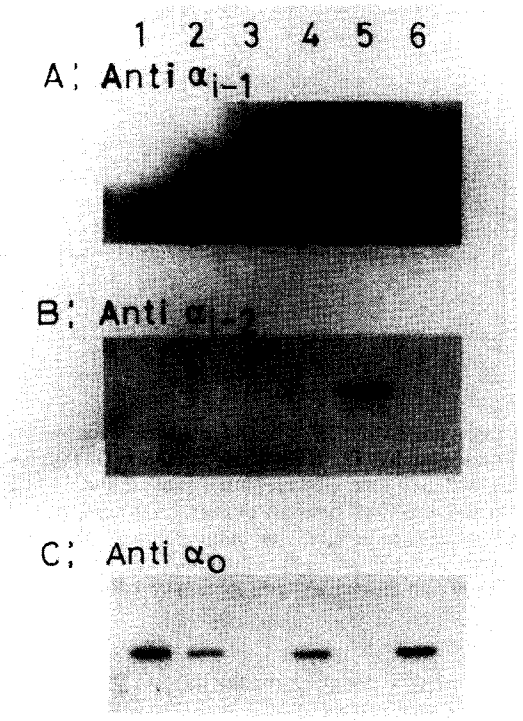

Fig.3. Immunoblot analysis of purified G-proteins with specific antibodies. The purified G-proteins were separated by SDS-polyacrylamide gel (12%) electrophoresis and analyzed for immunoblot as described in section 2.4. Lanes 1–6, peaks 1–6 shown in fig.1, respectively. (A and B) Antisera specific for the α-subunits of G_{i-1} and G_{i-2}, [13] respectively; (C) affinity-purified antibody specific for the α-subunit of G_o [6,11].

ribosylated by IAP in the presence of ^{32}P-labeled NAD (fig.2B), indicating that all the peaks (2–6) were indeed G-proteins serving as the substrate of IAP. The 39-kDa α of peak 1 also served as the substrate of IAP if $\beta\gamma$-subunits were added to the ribosylation mixture (fig.2B, lane 8). Any α of the 6 peaks was not ADP-ribosylated by cholera toxin even in the presence of ADP-ribosylation factor (ARF) [14] under a condition that purified G_s was certainly modified by the toxin (not shown).

We next studied how these 6 peaks were related to previously identified G-proteins by means of immunoblot analysis. As shown in fig.3A and B, specific antisera raised against synthetic decapeptides predicted from cDNAs encoding the α-subunits of G_{i-1} and G_{i-2} [13] reacted strongly with the 41- and 40-kDa polypeptides of peaks 3 and 5, respectively. The α of peak 3 also reacted weakly with specific antibody raised against synthetic peptides of the α of G_{i-3} [13], probably due to contamination of minute amounts of G_{i-3} in the G_{i-1} fraction of peak 3 (not shown). On the other hand, affinity-purified antibody raised against the purified α_{39} of G_o from rat brain [6,11] reacted strongly with the α-subunits of peaks 1, 2, 4 and 6 (fig.2C). These results indicated that peaks 1, 2, 4 and 6 eluted from Mono Q column contained G_o or G_o-like G-protein, and the peaks 3 and 5 contained G_{i-1} (with minute amounts of G_{i-3}) and G_{i-2}, respectively.

The different elution profiles observed among G_o and G_o-like peaks (1, 2, 4 and 6) might be due to different $\beta\gamma$-subunits tightly associated with their α-subunits. We, therefore, purified α-subunits from the heterotrimeric G-proteins in peaks 2 and 4 by means of incubation with ^{35}S-labeled GTPγS and Mg^{2+} [10]. Thus, the heterogeneity of G_o or G_o-like G-protein could be classified only based on the α-subunits. The radiolabeled GTPγS bound α-subunits obtained from peaks 2 and 4 (in the protein ratio of 2:1) were mixed with non-radiolabeled GTPγS bound α-subunit of peak 1 which had been prepared by incubation with non-radioactive GTPγS and Mg^{2+}. The mixture containing the three GTPγS bound α-subunits (two of them contained ^{35}S radioactivity in the amounts of 2:1) were applied to Mono Q column and then eluted with a linear NaCl gradient (fig.4). There were 3 major protein peaks containing α-subunits as shown by peaks *a*, *b* and *c* in fig.4; peak *d* appeared to be free GTPγS unbound to proteins, since there was no protein band in the fraction (not shown). The peaks *b* and *c* which retained ^{35}S-labeled GTPγS with the ratio of 2:1 were eluted from the column clearly distinct from the elution position of peak *a* containing no ^{35}S radioactivity. Thus, the 3 α-subunits among peaks 1, 2 and 4, which corresponded to peaks *a*, *b* and *c* in fig.4, respectively, appeared to be

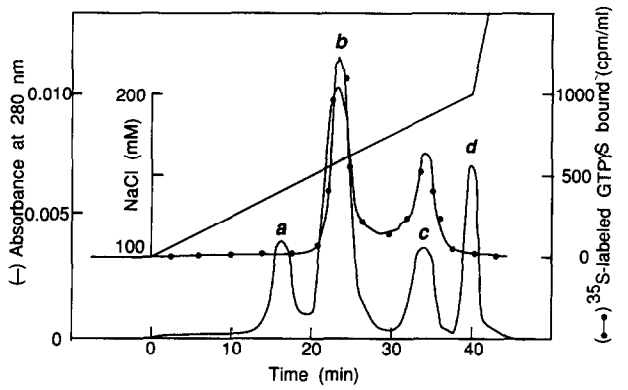

Fig.4. Elution profile of GTPγS bound α-subunits from Mono Q column chromatography. The mixture of ^{35}S-labeled GTPγS bound α-subunits which had been separated from peaks 2 and 4 in fig.1 and non-radiolabeled GTPγS bound α of peak 1 was applied to Mono Q column and eluted as in fig.1. The radioactivity of ^{35}S in the eluted fraction was measured (●—●). The absorbance at 280 nm of the eluted protein was also monitored (——).

different entities from one another. GTPγS-bound α-subunit of peak 6 was eluted from the anion-exchange column at a higher concentration of NaCl than those of peaks 1, 2 and 4 (not shown), so that the α-subunit of peak 6 was also distinct from the above 3 α-subunits.

In this report, we have succeeded in purifying several G-proteins serving as the substrate of IAP in addition to previously identified αβγ-heterotrimeric G-proteins from bovine brain membranes. Two of them, peaks 3 and 5 in fig.1, corresponded to the products of G_{i-1} and G_{i-2} cDNAs, respectively. The other proteins, peaks 1, 2, 4 and 6, appeared to be a family of G_o based on their immunological reactivities. Peak 4, which was the most abundant in the brain membranes, is very likely to be G_o previously identified in many neuronal tissues. The G_o-like protein in peak 2 of which α-subunit was slightly above 39 kDa on SDS-polyacrylamide gel (see fig.2A) would be the same entity as a G-protein, termed G_{39}^{*}, recently reported by Goldsmith et al. [9]. The other α-subunits of G_o-like proteins in peaks 1 (or *a* in fig.4) and 6 in fig.1, which were first identified in the present study, were apparently different from each other and also from G_o or G_{39}^{*}. Although it is not clear that these α-subunits of G_o-like proteins differ with respect to their primary sequences or post-translational modifications, the present paper is the first report showing that there is a heterogeneity in the α-subunit of G_o purified from brain membranes.

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