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Characterization of four G_0-type proteins purified from bovine brain membranes

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Recently we reported there were at least four types of G_0 or G_0-like proteins in bovine brain membranes based on their elution profiles from Mono Q columns and their immunological reactivities; one of the proteins was purified as an α-monomeric form, and the others as $\alpha\beta\gamma$-trimers. The four proteins, of which α-subunits were confirmed to be a family of G_0-type by an immunoblot analysis, were thus referred to as $\alpha_0 1$, $G_0 2$, $G_0 3$ and $G_0 4$, respectively, in order of their elutions from the column. Immunostained peptide mappings arising from proteolytic digestions of the four α-subunits, together with their fragmentation patterns containing radiolabeled ADP-ribose that had been incorporated by pertussis toxin-catalyzed ADP-ribosylation, suggested that the four G_0-α were classified into either of two groups such as $\alpha_0 1$ and $G_0 2$-α or $G_0 3$-α and $G_0 4$-α. The kinetic parameters of their GTPase activities, however, revealed that there were different properties between $\alpha_0 1$ and $G_0 2$-α or $G_0 3$-α and $G_0 4$-α. Thus, the four G_0-type proteins appeared to be different entities from one another.

GTP-binding protein; Pertussis toxin (IAP); GTPase activity

1. INTRODUCTION

GTP-binding proteins (G proteins) function as a transducer carrying signals from extracellularly oriented receptors to intracellular effectors in a variety of vertebrate cells [1]. The G protein has been identified as a heterotrimer with α-, β- and γ-subunits and was purified from the plasma membranes of various tissues. Identified and characterized members of this family include the stimulatory (G_s) and inhibitory(G_i) G proteins of the hormone-sensitive adenylate cyclase systems and transducin (G_t) of vertebrate retina that mediates rhodopsin-induced activation of cGMP-dependent phosphodiesterase. A fourth member of this family, termed G_o, has also been identified in brain tissues, though its precise function has not been classified.

The α subunits of G_i serve as the substrate of pertussis toxin- (IAP-) catalyzed ADP-ribosylation in their trimeric forms, have been classified into three subtypes of $\alpha_{i\text{-}1}$, $\alpha_{i\text{-}2}$ and $\alpha_{i\text{-}3}$ from analyses of molecular cloning of the genes [2] and cDNAs [3–6]. In regard to the α-subunits of G_o, at least two types of the genes were informed from Northern blot analysis [7]. Goldsmith et al. reported the purification of a novel type of G_o (termed G_{39}*) which might be a post-translational modification of G_o [8]. Lang suggested that there were two distinct G_o proteins based on the findings of their peptide mappings [9]. In the previous studies, we also reported there were four types of G_o (or G_o-like) proteins cross-reacting with a polyclonal anti-α_o antibody in bovine brain membranes [10]. However, it has not been clear that those α-subunits of G_o-type proteins differ with respect to their primary sequences or post-translational modifications. In this paper, the four purified G_o-type proteins are characterized by peptide mappings after their digestions with several proteases. The kinetic properties of the purified proteins are also examined in terms of the GTPase activity.

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2. MATERIALS AND METHODS

2.1. *Materials*

^{35}S-labeled guanosine 5′-*O*-(thio)triphosphate, GTPγS (44.4 TBq/mmol), [γ-^{32}P]GTP (1.1 TBq/mmol) and ^{32}P-labeled NAD (29.6 TBq/mmol) were purchased from DuPont-New England Nuclear. Biotinylated anti-rabbit IgG and Streptavidine horseradish peroxidase (HRP) were purchased from Amersham. Lysylendopeptidase and V8 protease were purchased from Takara Shuzou Co., Ltd. The sources of all other reagents used are those described in [10–12].

2.2. *Purification of four G_o proteins*

Four G_o proteins were purified from bovine brain membranes as described in [10]. The purified proteins were quantitated by staining with Amido black with bovine serum albumin as a standard protein [13]. G proteins were alkylated with *N*-ethylmaleimide (NEM) prior to electrophoresis as in [14]. Immunoblot analysis was performed as

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in [11,15,16]. Antibodies used in the present experiments were anti-α_o (A) which was affinity-purified rabbit polyclonal IgG raised against purified α_o and anti-α_o (B) which was also affinity-purified rabbit polyclonal antibody raised against synthetic oligopeptide corresponding to the predicted amino acid sequence (94–108; EYGDKERKADSKMVC) of α_o [3]. This region is specific for α_o and different from the other α-subunits of G proteins.

2.3. *Peptide mappings of four purified G_o proteins after their digestion with proteases*

The four purified G_o proteins were digested with lysylendopeptidase and V8 protease at the ratio of 50 (G proteins) to 1 (proteases) at 30°C for 20 h and 70 h, respectively. Prior to the digestions, the G_o proteins were denatured by heating at 100°C with 20 mM dithiothreitol for a few minutes and followed by incubation with 5 mM NEM for 30 min. The digested samples, after being electrophoresed, were transferred to polyvinylidene difluoride films. The films were incubated with various antibodies at 30°C for 12 h and treated with biotinylated anti-rabbit IgG and streptavidine HRP. The four purified G_o proteins were also ADP-ribosylated by IAP in the presence of ^{32}P-labeled NAD as in [10–12,14] and followed by digestions with lysylendopeptidase as described above. The samples were electrophoresed, and the radiolabeled polypeptides were detected by autoradiography.

2.4. *Assay of GTPase activity*

GTPase activity of the purified G_o proteins was assayed with various concentrations of [γ-^{32}P]GTP in a reaction mixture consisting of 20 mM sodium *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.4), 10 mM Na-EDTA and 35 mM $MgCl_2$ at 30°C as in [11,12]. The activities are expressed as turnover rates, which normalized the activity to the maximum amounts of ^{35}S-labeled GTPγS binding to G proteins as in [12].

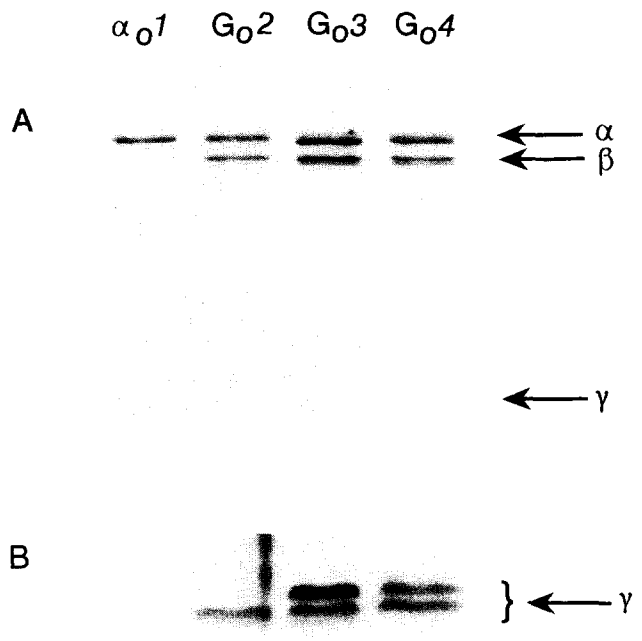

Fig. 1. SDS-PAGE of four G_o-type proteins purified from bovine brain membranes. (A) Three hundred ng of $\alpha_o 1$ or 650 ng of $G_o 2$, $G_o 3$ or $G_o 4$ were subjected to SDS-PAGE (12% gel) and stained with Coomassie blue. (B) The four G_o proteins were subjected to SDS-PAGE (15% gel) and stained with silver (only the region of γ-subunits is illustrated).

3. RESULTS AND DISCUSSION

We have recently reported that there are at least six distinct G proteins serving as the substrate for IAP-catalyzed ADP-ribosylation in bovine brain membranes [10]. Two of them were identified as $G_{i\text{-}1}$ and $G_{i\text{-}2}$ by immunoblot analysis with specific anti-$\alpha_{i\text{-}1}$ and anti-$\alpha_{i\text{-}2}$ antisera, and the other four cross-reacting strongly with specific anti-α_o IgG (A) were attributed to G_o-type proteins. For simplicity, the four G_o-type proteins were henceforth referred to as $\alpha_o 1$, $G_o 2$, $G_o 3$ and $G_o 4$, respectively, in order of their elutions from a Mono Q column chromatography, since one of the proteins was purified as an α-monomeric form, and the others were as $\alpha\beta\gamma$-trimers (see Fig. 1). $G_o 3$ was the most abundant in the brain membranes, suggesting that it must be identical to G_o previously identified in many neuronal tissues.

As shown in Fig. 1A, the four α-subunits of the purified proteins, of which molecular weights were all approximately 39000, exhibited mobilities slightly different from one another on SDS-PAGE; the larger order of their apparent molecular weights was $G_o 2$-α > $\alpha_o 1$ > $G_o 4$-α > $G_o 3$-α under the present conditions. There were, however, no apparent differences in the β-subunits among $G_o 2$, $G_o 3$ and $G_o 4$ based on their mobilities on SDS-PAGE. Fig. 1B shows silver-stained γ-subunits of the purified G proteins. There were at least two γ-subunits, and their distributions were not identical among the three trimeric G_o-type proteins; $G_o 3$ and $G_o 4$ had two major γ-subunits, while $G_o 2$ contained a single band of γ-subunits. Although $\beta\gamma$-subunits associated with each α-subunit were different from one another, the distinct elution profiles of the four G_o-type proteins from the Mono Q column were due to the properties of their α-subunits. The four α-subunits resolved from their $\beta\gamma$-subunits had been eluted separately from the column in manners similar to those observed with their trimeric forms [10].

The following studies performed by immunoblot analyses confirmed an idea that the above four α-subunits were not identical to one another, but they were indeed a family of G_o-type proteins. As illustrated in Fig. 2, all of the four α-subunits were similarly reacted with anti-α_o antibody (B) which was raised against synthetic oligopeptide (EYGDKERKADSK-MVC) corresponding to the predicted amino acid sequence (94–108) of α_o [3]. The sequence in α_o was the most heterogeneous region among G proteins [3], and the antibody did not crossreact with the other types of α-subunits, such as α_i, α_s, or α_t (data not shown).

When the four G_o-type proteins were digested with lysylendopeptidase or V8 protease and then analyzed by immunoblot with polyclonal anti-α_o IgG (A) which was raised against the purified α_{39} of G_o from rat brain [15,16], there were significant differences in the fragments immunoreacted with the antibody among the four α-subunits. The immunoreactive peptides cleaved

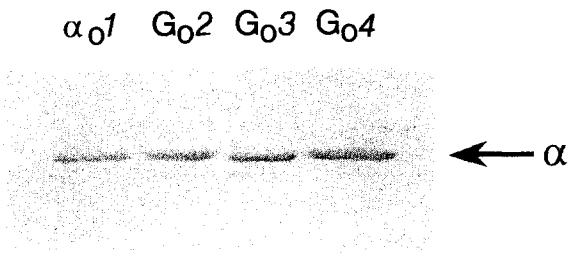

Fig. 2. Immunoblot analysis of the four G_o-type proteins. The four G_o-type proteins were separated by SDS-PAGE (12% gel) and analyzed by immunoblot with affinity-purified anti-α_o antibody (B) raised against synthetic oligopeptide corresponding to the predicted amino acid sequence (94–108; EYGDKERKADSKMVC) of α_o as described in section 2.2.

from α_o1 or G_o2-α were apparently different from those from G_o3-α or G_o4-α upon their digestion with lysylendopeptidase (Fig. 3A). Such findings were also confirmed by peptide mappings resulting from their digestions with V8 protease (Fig. 3B). However, no apparent differences were observed between α_o1 and G_o2-α or G_o3-α and G_o4-α in their digested patterns by either lysylendopeptidase or V8 protease. Similar findings were also observed when the four G_o-type proteins were digested with other proteases, such as trypsin, chymotrypsin or arginylendopeptidase, and then immunostained with anti-α_o IgG (A) (data not shown).

The four G_o-type proteins that had been ADP-ribosylated by IAP in the presence of ^{32}P-labeled NAD were also digested with lysylendopeptidase and V8 protease, and their cleavage products containing the radioactivity are illustrated in Fig. 3C, D, respectively. Since the ADP-ribosylation of α-subunits by IAP occurred at cysteine residue near their carboxyl termini, the radiolabeled fragments included the regions of their carboxyl termini. The cleavage patterns containing the carboxyl terminus of α_o1 or G_o2-α was apparently different from that of G_o3-α or G_o4-α upon their digestions with lysylendopeptidase or V8 protease. Again, there were no significant differences in the radiolabeled fragments between α_o1 and G_o2-α or G_o3-α and G_o4-α. These results suggested that four G_o proteins were classified into either of two groups in terms of their proteolytic digestion patterns; such were α_o1 and G_o2-α as the first group, or G_o3-α and G_o4-α as the second one.

In order to further clarify the differences among the four G_o-type proteins, we next investigated their GTPase activities, and the results are summarized in Table I. Since α_o1 was purified as an α-monomer, $\beta\gamma$-subunits purified from G_o mixture were added to form the $\alpha\beta\gamma$-trimer (termed G_o1). The GTPase activities of the four G_o-type proteins displayed essentially similar kinetic properties in terms of K_m for GTP and V_{max} (the steady-state rate of GTP hydrolysis) to one another.

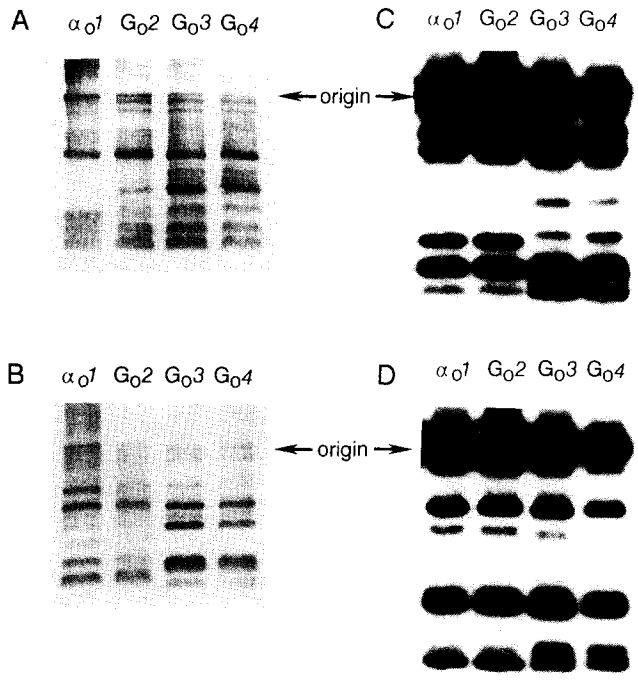

Fig. 3. Immunoblot analysis of the four G_o-type proteins after protease digestions. (A,B) The four G_o-type proteins were digested with lysylendopeptidase (A) or V8 protease (B) and then subjected to SDS-PAGE (15% gel). Immunoblot analyses were performed with anti-α_o IgG (A) as described in 2.3. (C,D) The four G_o-type proteins were ADP-ribosylated by IAP in the presence of ^{32}P-labeled NAD and then digested with lysylendopeptidase (C) or V8 protease (D). These reaction mixtures were subjected to SDS-PAGE (15% gel) and analyzed by autoradiography.

However, differences were noted in the special comparison between G_o1 and G_o2 or G_o3 and G_o4. The K_m and V_{max} values obtained with G_o2 (or G_o4) were slightly though significantly higher than those observed with G_o1 (or G_o3). Thus, the two α-subunits of the first (α_o1 and G_o2-α) or the second (G_o3-α and G_o4-α) groups which were indistinguishable from each other in terms of their peptide mappings could be classified into further two distinct entities with respect to their GTPase properties.

Table I

Kinetic parameters for GTPase activities of four purified G_o-type proteins

G_o proteins	GTPase activity	
	K_m (M)	V_{max} (min^{-1})
$\alpha_o1+\beta\gamma$	4.9×10^{-7}	0.57
G_o2	8.0×10^{-7}	0.73
G_o3	5.0×10^{-7}	0.46
G_o4	1.3×10^{-6}	0.73

GTPase activities of the four purified G_o-type proteins were assayed with various concentrations of [γ-^{32}P]GTP as described in section 2.4. The K_m and V_{max} values were obtained from data analyzed by the Lineweaver-Burk plots of their GTPase activities

In the present study, we characterized four G_o-type proteins purified from bovine brain membranes by means of peptide mappings after their proteolytic digestions and kinetic properties of their GTPase activities. All the four proteins served as the substrate for IAP-catalyzed ADP-ribosylation and had a GTPase activity characterized by usual $\alpha\beta\gamma$-trimeric GTP-binding proteins, suggesting that they were existent in the native brain membranes and not artificial products due to proteolysis or denaturation during the purification. The two groups of G_o-α in terms of their peptide mappings, such as $\alpha_o 1$ or $G_o 2$-α and $G_o 3$-α or $G_o 4$-α, might be attributed to the two types of α_o genes by Northern blot analysis [7]. Although it is still unclear how the α-subunits of four G_o-type proteins differ in terms of their primary amino acid sequences or post-translational modifications, the present paper is the first report dealing with the characteristics of heterogeneous α-subunits of G_o-type proteins structurally and functionally. Interactions of these G_o proteins with specific receptors and/or effectors, if present, are important in questions on signal-coupling proteins.

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