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Separation of two βγ subunit complexes of brain GTP-binding proteins composed of distinct γ subunits

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The γ subunits of GTP-binding proteins are always associated with β subunits under physiological conditions, and at least two γ subunits exist in the brain. We report here that brain βγ subunit complexes composed of distinct γ subunits can be separated by anion exchange chromatography under nondenaturing conditions. One βγ complex was composed of a 36-kDa β subunit and a 6-kDa γ subunit and the other was composed of 36/35-kDa β subunits and 4.5-kDa γ subunit. The 6-kDa γ subunit was phosphorylated by protein kinase C but the 4.5-kDa γ subunit was not.

GTP-binding protein; γ Subunit; Phosphorylation; Protein kinase C

1. INTRODUCTION

GTP-binding proteins (G proteins) are considered to function as signal transducers between membrane-bound receptors and intracellular effectors [1]. These G proteins are heterotrimers composed of three different subunits (α, β and γ). The α subunits, which bind and hydrolyze GTP, are unique among G proteins, while the β subunits, which are associated with the γ subunits under physiological conditions, are seem to be common [1]. Because the α subunit appeared to specify the function of a particular G protein, most research has focused on α subunits. Therefore, the role of βγ subunit complex has been less clear. The major role of βγ complex seems to make the α subunit inactive form by its association. There are indications that the βγ complex might act independently on effectors [2]. It was also reported that the βγ complex was required for the interaction of the α subunit with the receptor [3].

Two β subunits have been identified by isolating cDNAs, one coding for a 36-kDa protein and another for a 35-kDa protein [4–6]. The γ subunits are known to be composed of multiple molecules [7–11], but have been poorly characterized. The primary structures of a γ subunit of transducin (G_t) [12–14] and that of G_i and G_o [15,16] have been revealed recentely, and they have 55% homology. Robishaw et al. [16] isolated two γ subunits (6-kDa and 5-kDa) from the denaturing G proteins of bovine brain, and showed their difference in the sequence of peptides prepared by partial proteolysis [16]. However, it has not yet been possible to isolate individual βγ complex which still retains activity. Recently, Fukada et al. separated two βγ complexes of transducing composed of distinct γ subunits by anion exchange chromatography under nondenaturing condition [17]. We show here the isolation of two βγ complexes of brain G proteins composed of distinct γ subunits which retain activities.

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2. MATERIALS AND METHODS

2.1. *Materials*

[^{32}P]ATP (25 Ci/mmol) was purchased from ICN Radiochemicals. Diolein and brain extract (Folch fraction I) were obtained from Sigma Chemical Company. Protein kinase C, which was purified from porcine brain (I + II) [18], was kindly supplied by Dr. T. Haga, Tokyo University.

2.2. *Isolation of βγ subunit complexes*

The βγ subunit complexes were purified from bovine cerebral cortex as described previously [19]. In brief, pertussis-toxin substrate G proteins were purified from the cholate extract of bovine brain membranes by the use of DEAE-Sephacel and Ultrogel AcA 34 chromatography. Then G proteins were applied to heptylamine-Sepharose column and the α and βγ subunits were separately eluted with the buffer containing 20 μM $AlCl_3$, 6 mM $MgCl_2$ and 10 mM NaF (AMF). The fractions enriched with βγ complexes were applied to a DEAE-Sephacel column and eluted with the gradient of NaCl (0–150 mM) in 20 mM Tris-HCl (pH 8.0)/0.1 mM EDTA/0.3% Lubrol PX (Buffer A) containing 5 mM β-mercaptoethanol and AMF. The βγ fractions free from α subunits were pooled. The purified βγ fraction was diluted with 2 vols of Buffer A and then loaded on a TSK-GEL DEAE-5PW (0.8 × 7.5 cm, Tosoh) column which had been equilibrated with Buffer A. The column was washed with 50 mM NaCl in Buffer A and eluted at a flow rate of 1 ml/min with a linear gradient (25 ml) from 50 to 200 mM NaCl in Buffer A using a Pharmacia FPLC system. Each fraction (1 ml) was collected in a tube which contained 10 μl of 100 mM dithiothreitol. The elution profile is shown in Fig. 1. Fractions 13–17 and fractions 22–24 were pooled, rechromatographed on the same column.

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2.3. *Electrophoresis*

Sodium dodecyl sulfate-polyacrylamide (10%) gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli [20]. 8 M urea/SDS-polyacrylamide (12.5%) gel electrophoresis (urea/SDS-PAGE) was performed by the method of Swank and Munkres [21]. As molecular weight standards, low molecular weight (LMW) and polypeptide molecular weight (PMW) Electrophoresis Calibration Kits from Pharmacia were used for SDS-PAGE and urea/SDS-PAGE, respectively.

2.4. *Phosphorylation of βγ subunit complexes*

Phosphorylation of βγ subunit complexes with protein kinase C was carried out by the method of Haga et al. [18]. The βγ complexes (2.5 μg) in the Lubrol PX solution were incubated with protein kinase C preparation (0.025 unit; one unit is defined as the mount of enzyme that incorporates 1 nmol of phosphate from ATP into histon per minute at 30°C) in a medium containing 10 μM [^{32}P]ATP (1000 cpm/pmol), 0.5 mM $CaCl_2$, 5 mM Mg acetate, 10 μg of brain extract and 0.2 μg of diolein (total volume, 70 μl) at 30°C for 1 h. After incubation, the reaction mixtures were dialyzed against 10 mM H_3PO_4-Tris (pH 6.8)/0.1 mM EDTA/5 mM β-mercaptoethanol/0.05% SDS at 4°C for 4 h, and were freeze-dried. The samples were incubated in the sample buffer containing 50 mM H_3PO_4-Tris (pH 6.8)/ 1% SDS/1% EDTA/1% β-mercaptoethanol/8 M urea at 60°C for 10 min and subjected to urea/SDS-PAGE. Then the gel was stained with silver and dried. The dried gel was autoradiographed at −80°C using Fuji x-ray film RXO-H.

3. RESULTS AND DISCUSSION

The purified βγ subunit complexes of bovine brain was subjected to DEAE-5PW column chromatography and the elution profile is shown in Fig. 1A. First, the small shoulder appeared and then two peaks and the last shoulder were eluted. SDS-PAGE analysis (Fig. 1B) revealed that each fraction contained both β and γ subunits. All fractions contained 36-kDa β, and 35-kDa β was eluted in the latter fractions. Consequently, early fraction contained only 36-kDa β subunits. The same fractions were subjected to urea/SDS-PAGE for a further analysis of γ subunit (Fig. 1C). Urea/SDS-PAGE analysis showed that brain γ subunit was composed of two components with apparent molecular weight values of about 6000 and about 4500, which are now referred to as γ-1 amd γ-2, respectively. The complex of γ-1 and β or that of γ-2 and β was named βγ-1 or βγ-2, respectively. As shown in Fig. 1C, γ-1 and γ-2 were partially separated on this DEAE-5PW chromatography. The first small shoulder corresponds to βγ-1 and the last shoulder to βγ-2 but the major two peaks contained both γ-1 and γ-2. To isolate both βγ-1 and βγ-2, the early eluates (fraction 13–17) and the late eluates (fraction 22–24) from DEAE-5PW column were rechromatographed on the same column, respectively. Fig. 2 shows SDS-PAGE and urea/SDS-PAGE analyses of the final preparations. The βγ-1 was composed of 36-kDa β and 6-kDa γ and βγ-2 was composed of 36/35-kDa β and 4.5-kDa γ.

Because the apparent molecular weights of the γ-1 (6000) and γ-2 (4500) are in good agreement with those (γ_6; 6000, γ_5; 5000) obtained by Robishaw et al. [16], it is clear that the γ-1 and γ-2 correspond to γ_6 and γ_5,

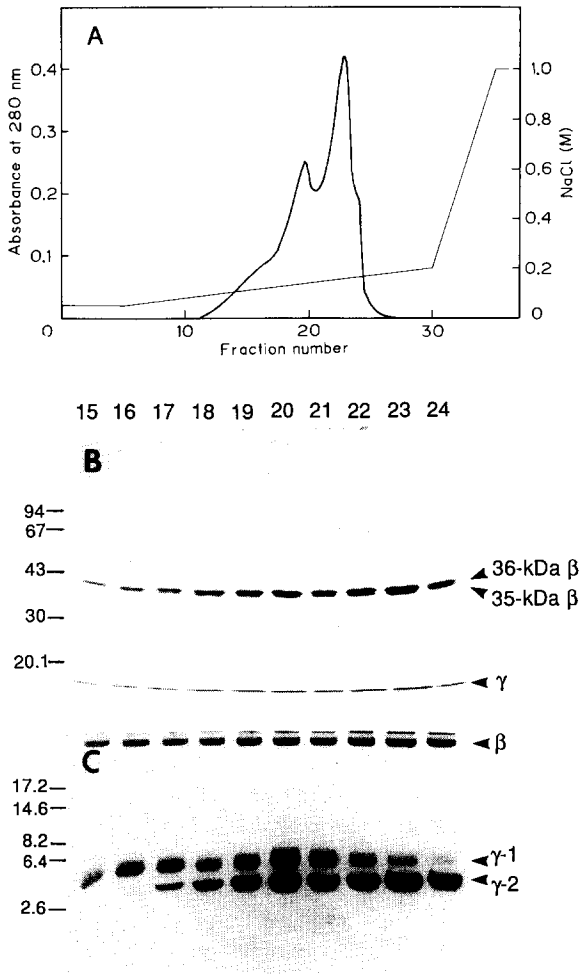

Fig. 1. TSK-GEL DEAE-5PW column chromatography of brain βγ subunit complexes. (A) The purified βγ complexes of bovine brain (2 mg of protein) was loaded on the TSK-GEL DEAE-5PW column and eluted as described in section 2. Thick line, absorbance at 280 nm; thin line, NaCl concentration. (B) SDS-PAGE patterns of the column fractions stained with Coomassie brilliant blue. (C) 8 M urea/SDS-PAGE patterns of the column fractions stained with silver. Number on the left indicate molecular mass in kDa.

respectively. Robishaw et al. demonstrated that the γ_6 and γ_5 subunits had different primary structures [16]. The apparent molecular weight of γ-1 estimated from the electrophoretic mobility was in disagreement with the molecular weight expected from the cDNA sequence (7850) [16]. Such disaggreement in the apparent molecular weight was also reported in γ subunit of G_t [17].

Two βγ subunit complexes of G_t revealed a marked difference in their enhancement of guanine nucleotide binding to the α subunit in the presence of a photobleaching intermediate of rhodopsin [17]. To examine whether the βγ-1 and βγ-2 of brain G proteins also display such functional differences, we tested various activities of the βγ subunit complexes, such as the effect of βγ on the ADP-ribosylation of *G* protein α

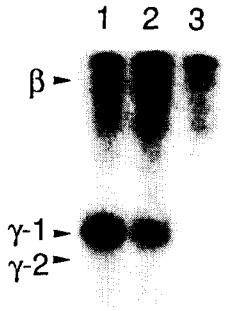

Fig. 2. SDS-PAGE and urea/SDS-PAGE patterns of βγ-1, βγ-2 and their mixture. The βγ-1 and βγ-2 rich fractions of DEAE- 5PW chromatography (Fig. 1) were rechromatographed on the same column. The βγ-1 (lane 1) and βγ-2 (lane 3) fractions obtained from the rechromatography and their mixture (lane 2) obtained from the frist chromatography (Fig. 1, fraction 18–21) were subjected to SDS-PAGE (A) and urea/SDS-PAGE (B).

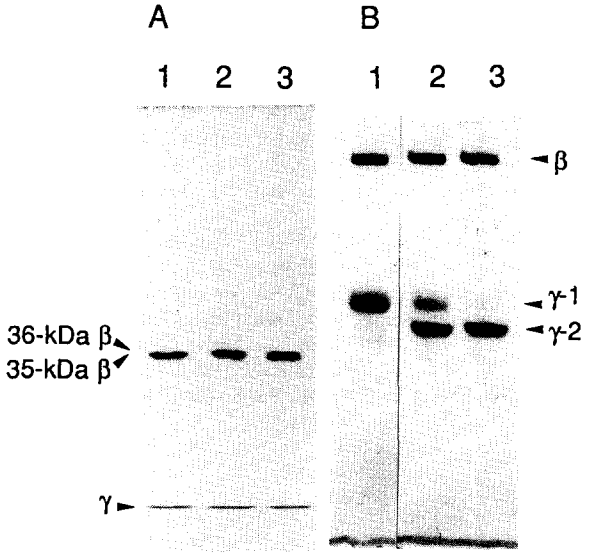

Fig. 3. Phosphorylation of the γ subunit by protein kinase C. The experimental conditions are given under section 2. Lane 1, βγ-1; lane 2, the mixture of βγ-1 and βγ-2; lane 3, βγ-2.

subunits by pertussis toxin [22], the inhibition of calmodulin-dependent cyclic AMP phosphodiesterase by βγ [23] and the activation of $G_o\alpha$ in the presence of either βγ by mastoparan [24]. However, no significant difference between βγ-1 and βγ-2 was found in these experiments (not shown). These results suggest that there is no difference between βγ-1 and βγ-2 in the ability to interact with α subunits, calmodulin and mastoparan in the presence of α subunit.

Hage et al. [18] reported that γ subunits of porcine brain G_{i1} and G_o were phosphorylated by protein kinase C but was not the γ subunit of G_{i2}. Their results suggested that either γ-1 or γ-2 might be phosphorylated by protein kinase C. To examine this possibility, the βγ-1, βγ-2 and their mixture were treated with porcine brain protein kinase C and then subjected to urea/SDS-PAGE. The autoradiogram showed that γ-1 was phosphorylated by protein kinase C but was not γ-2 (Fig. 3). The molar amount of incorporated phosphate was estimated to be 0.12 for γ-1, when βγ-1 was incubated with 10 μM [^{32}P]ATP and protein kinase C for 60 min at 30°C. The reason for the low incorporation of phosphates to the γ-1 was not clear. The addition of two times of protein kinase C preparation did not increase the incorporation, suggesting that the amount of the kinase was sufficient. Because the treatment of βγ-1 with alkaline phosphatase did not increase the phosphorylation of γ-1 by protein kinase C, the low incorporation of phosphate did not seem to be due to the presence of endogenously phosphorylated form of γ-1. Haga et al. [18] demonstrated that the β subunits were phosphorylated in the presence of α subunits in phospholipid vesicles, but not in the cholate solution. The β subunits was not phosphorylated in the present study in which the phosphorylation was carried out in the Lubrol solution, but the β in some preparations of βγ-2 or the mixture of βγ-1 and βγ-2 was phosphorylated in the same conditions (data not shown). Some small amount of contaminants including α subunits or/and the difference in detergent used might influence the phosphorylation of *G* proteins by protein kinase C. Protein kinase A did not phosphorylate either γ subunit.

In this report, we have separated two βγ subunit complexes of bovine brain composed of distinct γ subunits. Only γ-1 was phosphorylated by protein kinase C, but the physiological significance of this phosphorylation is not yet known.

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