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TWO γ-SUBUNITS, γ-I AND γ-II, COMPLEX WITH THE SAME β-SUBUNITS IN BOVINE BRAIN G-PROTEINS (Gi/o)

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When a mixture of bovine brain G-proteins (Gi/o) was loaded onto an octyl sepharose column in the presence of AlF_4^-, α-subunits of molecular weights 39 kDa and the 41 kDa were eluted separately, followed by the appearance of two distinct peaks containing βγ-subunits (βγ-I, βγ-II). Both βγ-I and βγ-II possessed identical β-subunits but different γ-subunits. The molecular weights of the two γ-subunits determined by SDS-polyacrylamide gel electrophoresis both in the presence and absence of urea were 4.5 kDa (γ-I) and 5.0 kDa (γ-II). Tests indicated that the two isolated γ-subunits are intact and have not undergone proteolysis. The amino acid composition of γ-I appeared to be distinct from that of γ-II. Therefore, this method is a simple procedure for isolating βγ-I and βγ-II.

G-proteins couple to various membrane receptors and transduce signals to specific effector systems in cells [1-3]. The G-protein family consists of α-, β- and γ-subunits. The α-subunit can bind a GTP and has GTPase activity. In G-proteins, there are variations of α-subunits. These heterogeneous α-subunits have been characterized to determine G-protein functions, such as specific coupling either between a G-protein and a receptor, or a G-protein and an effector. In contrast to the observed diversity of the α-subunit, cDNAs encoding β-subunits from various mammalian tissue types share high homology [4-7]. Four cDNAs encoding the γ-subunits have been isolated from different mammalian sources [8-11]. These γ-

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Abbreviations used: G-protein, guanine nucleotide-binding regulatory protein; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis.

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subunit cDNAs show much less homology with one another compared with the high homology among the β-subunit cDNAs. At present, no biological significance for the diverse β- and γ-subunits has been elucidated. Uncoupled β- and γ-subunits have no physiological activity. Therefore, in order to study the physiological activity of heterogeneous β- and γ-subunits, they must be isolated in the βγ complex state. Recently, Asano et al. [12] were able to isolate two kinds of βγ-subunits with distinct γ-subunits (βγ-1 and βγ-2) from Gi/o.

We report here an effective method for separating two kinds of bovine brain βγ complexes (βγ-I and βγ-II) from Gi/o under nondenaturing conditions utilizing an octyl sepharose chromatography. Electrophoretic analyses of βγ-subunits showed that only the γ-subunits are distinct (5.0 kDa for γ-I and 4.5 kDa for γ-II), and γ-I and γ-II each complexes with both the 35 kDa and 36 kDa β-subunits. This contrasts with the findings of Asano et al. [12], in which γ-1 complexes with only the 35 kDa β-subunit, and γ-2 with both the 35 kDa and 36 kDa β-subunits.

Experimental Procedures

Preparation of G-proteins: The Gi/o mixture was prepared from bovine brain by the method of Sternweis and Robishaw [13], with some modifications by Milligan and Klee [14] and Newton and Klee (unpublished data); the pooled DEAE-sephacel fractions were concentrated by ultrafiltration using an Amicon PM-30 membrane.

Separation of βγ-subunits from α-subunits: The Gi/o mixture was dialyzed against buffer A (20 mM Tris, pH 8.0, 0.3 % cholate, 0.1 M NaCl, 6 mM $MgCl_2$, 1 mM EDTA, 10 mM NaF, 10 μM $AlCl_3$ and 1mM DTT), then loaded onto an octyl sepharose column equilibrated with buffer A. After the column was washed with buffer A, G-protein subunits were eluted concomitantly with linear gradients of NaCl and cholate, from 0.1 M to 0.05 M and from 0.3 % to 1.2 %, respectively (Fig. 1A).

Methylation of γ-subunit: The methylation of Gγ was performed with S-adenosyl-[methyl-^{3}H]-methionine by the method of Fung et al. [15]. Rat brain microsomal fractions were prepared by the method of Fung et al. [15].

Isolation of γ-subunit: The purified βγ-I was dialyzed against buffer B (8 M urea, 20 mM Tris, pH7.5, 1 mM EDTA, and 1 mM DTT), then loaded onto a DEAE-sephacel column equilibrated with buffer B. The column was washed with buffer B. The γ-I eluted in the wash fraction. The column was then washed with linear NaCl gradient (0 to 0.15 M), with the β-subunits (35 and 36 kDa) eluted at about 0.1 M NaCl. The procedure for the purified βγ-II complex was the same as for βγ-I, with the same results.

Others: Amino acid compositions were determined with a Hitachi 835 amino acid analyzer. Polyacrylamide gel electrophoresis (PAGE) in the presence of 0.1%

SDS was performed by the method of Laemmli [16]. PAGE in the presence of both urea and SDS was performed by the method of Swank and Munkres [17]. Two-dimensional gel electrophoresis was performed by the method of Backlund et al. [18]. Protein concentration was determined by the method of Lowry [19] or from the absorption at 280nm using extinction coefficients of 0.93 for 1 mg/ml α-subunit, and 1.47 for 1 mg/ml βγ-subunit.

Results and Discussion

Heptylamine sepharose and AlF_4^- are widely used for separating α- and βγ-subunits of GTP-binding proteins with each other [20]. Using anion exchange column chromatography, two forms of the βγ complex, βγ-1 and βγ-2, were characterized from isolated βγ-subunits by Asano et al. [12]. Their results suggest that one species, βγ-1, contains a 6 kDa γ-subunit and a single 35 kDa β-subunit, while the other species, βγ-2, has a 4.5 kDa γ-subunit and two forms of the β-subunit (35/36 kDa). However, the separation of the two βγ-subunits, reported by Asano et al. [12], appears to be incomplete on that chromatography. Thus, we tried another method for separating the subspecies of the βγ-subunits. The purified G-proteins (Gi/o), αβγ complex, from bovine brain were loaded onto an octyl sepharose column in the presence of AlF_4^-, and the elution was performed by the linear gradients of NaCl and cholate. The elution profile and a SDS-PAGE of selected fractions are shown in Fig. 1, revealing good separation of the α- and the βγ-subunits. The first two small peaks correlate with the 39 kDa and 41 kDa α-subunits, and the following two distinct peaks (Peak 1 and Peak 2) with the βγ-subunits (βγ–II and βγ–I). The β-subunits of both βγ–I and βγ–II separated into a doublet of 36 kDa and 35 kDa (Fig. 1**B**), while the molecular mass of γ–I is slightly larger than that of γ–II. Pooled fractions containing βγ–I (Peak 2, Fig. 1**A**) were subjected to: 1) SDS-PAGE with urea (Fig. 2**A**, lane 1) and 2) two-dimensional gel electrophoresis (Fig. 2**B**, panel **a**). The same was done with the βγ–II pooled fractions (Fig. 2**A**, lane 2, and Fig. 2**B**, panel **b**). The molecular masses of γ–I and γ–II were estimated to be 5.0 kDa and 4.5 kDa, respectively (Fig. 2**A**). Only the β-subunits were resolved using two-dimensional gel electrophoresis (30-43 kDa range) (Fig. 2**B**). The two-dimensional chromatograms of βγ–I and βγ–II appear to be identical (*i.e.*, relative intensities, mobilities and pIs of three resolved proteins). Therefore, both βγ–I and βγ–II contain the same species of the β-subunits. Thus,

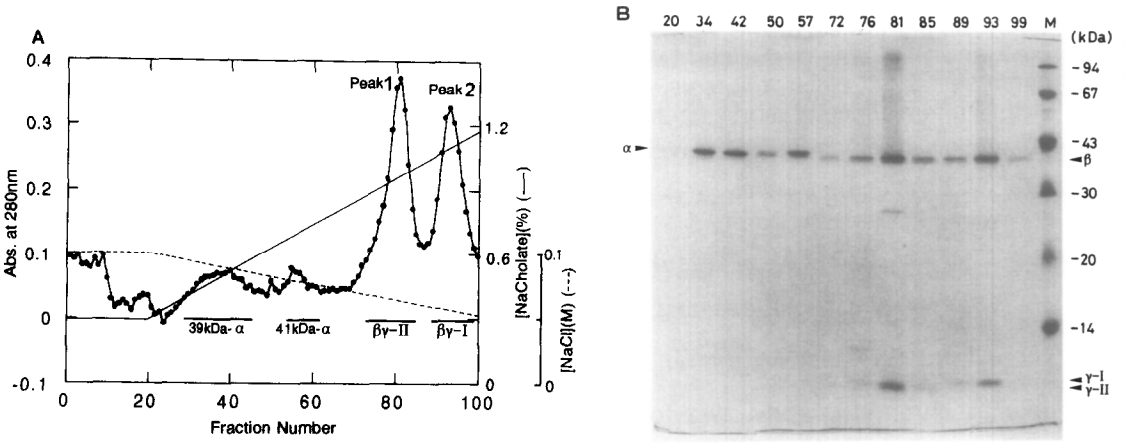

Fig. 1. An elution profile of the octyl sepharose column chromatography. **A**, After dialysis against buffer A ("Experimental Procedures"), 30 mg of the Gi/0 mixture was loaded onto a 100 ml octyl sepharose column equilibrated with buffer A. The column was washed with buffer A (30 ml/h flow rate, 5ml fraction volume) for 20 fractions, then 400 ml-linear gradients of both NaCl (0.1-0.05 M) and cholate (0.3-1.2 %) were used. Solid bars under each of the four peaks indicate pooled fraction numbers and are labeled with the appropriate subunit(s). **B**, SDS-PAGE (15% gel) pattern after staining with Coomassie Brilliant Blue of the selected fractions from **A** is shown. The numbers shown on the top of the gel indicate fraction numbers, and M is the lane containing the molecular weight standards. Relative positions of α-, β, and γ-subunits are also shown.

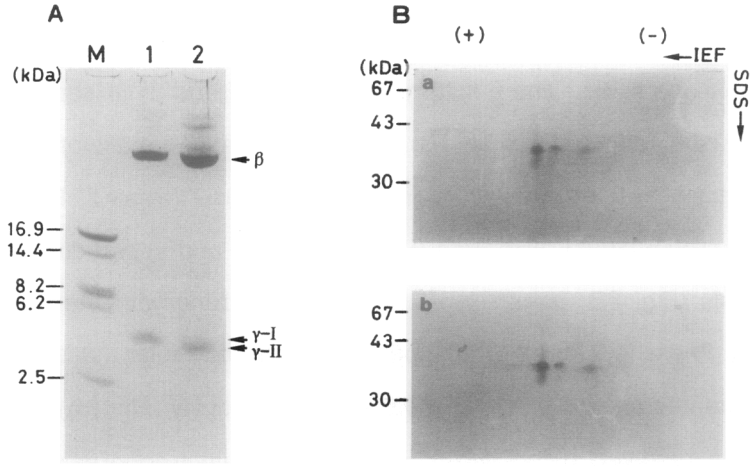

Fig. 2. Pooled fractions of βγ-I (Peak 2 of the octyl sepharose column) were subjected to : **A** (lane 1), PAGE (12.5 %) in the presence of both 0.1 % SDS and 8M urea, **B** (panel **a**), two-dimensional PAGE, first dimension IEF, pH 5-7, second dimension, SDS-PAGE (15 % gel). Results with βγ-II pooled fractions (Peak 1) are shown in lane 2 and panel **b**. **A**, the pattern stained with Coomassie Brilliant Blue; **B**, with silver. Lane M contains the molecular weight standards.

octyl sepharose column chromatography in the presence of AlF_4^- was shown to be an effective method to separate subspecies of α- and βγ-subunits of bovine brain G-proteins (Gi/o). The apparent molecular masses of the γ-I (5.0 kDa) and γ-II (4.5 kDa) are in agreement with those reported by Robishaw et al. [11] and Asano et al. [12] (6 kDa for γ-1 and 4.5 kDa for γ-2). However, the β-subunit composition of the βγ complexes isolated by Asano et al. [12] differs from those reported on here. Therefore, the βγ-1 and βγ-2 characterized by Asano et al. [12] may not be identical with βγ-I and βγ-II characterized in this paper - thus the slight difference in nomenclature (βγ-1 - βγ-I).

The observation of diversity between G-protein γ-subunits, γ-I and γ-II, is similar to that of γ-subunit of the the photoreceptor G-protein (Tγ), which consists of Tγ-1 and Tγ-2 [21]. Fukada et al. reported that the Tγ-2 has a posttranslationally S-farnesylated and/or methylesterified cysteine on the COOH-terminal, while such a modified cysteine residue is absent in Tγ-1 [22]. Interestingly, as determined from their respective cDNAs, both Tγ and Gγ have a characteristic COOH-terminal sequence, CAAX motif (in which C represents cysteine, A aliphatic and X any amino acid) indicative of posttranslational isoprenylation and truncation of AAX [23, 24]. In fact, it has been shown that the γ-subunits of Gi/o are isoprenylated, *i.e.*, S-geranylgeranylated [25, 26], and methylesterified [15] at the cysteine on the COOH-terminal. Tγ-1 and Tγ-2 have identical amino acid sequences, with the exception that Tγ-2 has one (or two) extra residue(s) at the COOH-terminal and contains a S-farnesylated cysteine at the COOH-terminal [27]. The similarity between Tγ (Tγ-1 and Tγ-2) and Gγ (γ-I and γ-II) may suggest that either γ-I or γ-II does not contain the modified COOH-terminal cysteine. In order to characterize the COOH-terminal structures of the γ-subunits, the carboxyl methylation of both Gγ-subunits was examined using the method of Fung et al. [15]. βγ-I and βγ-II were incubated with rat brain microsomal fractions and S-adenosyl-[methyl-^{3}H]-methionine. As shown in Fig. 3, ^{3}H from S-adenosyl-[methyl-^{3}H]-methionine was abundantly incorporated into both γ-I and γ-II. This result indicate that both γ-I and γ-II were methylesterified with carboxylmethyltransferase present in microsomal fractions. Stephenson et al. [28] clearly showed that isoprenylation of the sulfhydryl group is required for methyl esterification of the COOH-terminal cysteine. Therefore, it is

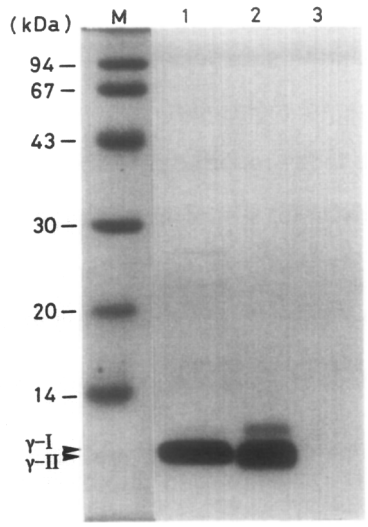

Fig. 3. Methylation of γ-subunits by an intrinsic methyl transferase present in the microsomal fractions in rat brain. 0.04 mg/ml βγ-I (lane 1), βγ-II (lane 2) and control (no βγ-subunits, lane 3) were incubated in 100 mM sodium phosphate, pH 7.0, 0.1 M NaCl, 5 mM $MgCl_2$, 10 μM S-adenosyl-[methyl-^{3}H]methionine (300 mCi/mmol), 0.5 mM DTT, and 2 mg/ml rat brain microsomal fractions at 37°C for 2 hours. After incubation, the reaction was stopped by adding SDS to 1%. Samples (10 μl each) were then subjected to SDS-PAGE (15 % gel). The fluorogram of the dried gel is shown. Lane M contains the molecular weight standards stained with Coomassie Brilliant Blue.

strongly suggested that both γ-I and γ-II, which were mehtylesterified, have isoprenylated cysteines on the COOH-terminals. A low radioactive band with slightly larger molecular mass than that of γ-II is observed on the lane 2. Incubation with the microsomal fractions could modify γ-II, or a minor contaminant, which was methylated, might be present in βγ-II. Attempts to characterize NH_2-terminal of both γ-subunits failed, as no PTH-amino acid by the Edman degradation was detected, presumably due to the NH_2-terminal modification. All of this data suggests that neither γ-I nor γ-II is a proteolytic product.

In order to compare the structure of the two γ-subunits, we isolated γ-I and γ-II using DEAE-sephacel column chromatography (see experimental procedures), and purified with C8 reverse phase column equipped with HPLC (data not shown). Amino acid analyses of the purified γ-I and γ-II were performed. As shown in Table 1, the amino acid composition of γ-I appears to be distinct from that of γ-II. It is clear that γ-I is almost identical with the composition from the deduced amino

Table 1. Amino acid compositions of isolated G protein γ-subunits

Amino acid residues	No. of residues/protein		
	Gγ-I	Gγ-II	Predicted from cDNA[10, 11]
D/N	6.5	5.1	7
T	2.1	1.6	2
S	4.2	5.5	4
E/Q	7.7	8.2	8
G	0	2.5	0
A	12.0	6.0	13
V	2.9	3.1	3
M	1.9	1.0	2
I	2.9	3.4	4
L	4.9	4.5	6
Y	1.3	1.0	1
F	2.8	1.1	3
K	6.6	5.5	7
H	1.0	0.8	1
R	2.9	3.7	3
P	4.7	4.5	4
C	ND*	ND*	2

HPLC purified Gγ-I (400 pmol) and Gγ-II (300 pmol) were hydrolyzed in 6 N HCl at 110 °C for 24 hours.
* ND, not determined.

acid sequence from the γ-subunit cDNAs described [10, 11]. Amino acid sequencing is necessary to further clarify any primary sequence differences. Possible functional differences of the various βγ-subunits also must to be examined.

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