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A brain-specific γ subunit of G protein freed from the corresponding β subunit under non-denaturing conditions

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Abstract

It has generally been accepted that the β and γ subunits of G proteins are tightly associated and can only be dissociated under denaturing conditions. However, one form of the γ subunit, free of the β subunit, was isolated under non-denaturing conditions during the purification of $\beta\gamma$ complexes from bovine brain. Amino acid sequence analysis revealed that the N-terminus of this γ subunit was not blocked and its sequence was identical to that of a brain-specific γ subunit, γ_3. Among three forms of γ subunits observed in bovine brain (γ_2, γ_3 and γ_7), γ_3 was the only form that was not modified at the N-terminus. Although the physiological significance of the free form of γ_3 is not clear, these results suggest that N-terminal modification of γ subunits may be important for interaction with β subunits.

Key words: G protein; $\beta\gamma$ Complex; γ Subunit; Bovine brain

1. Introduction

Guanine nucleotide-binding regulatory proteins (G proteins) couple a variety of cell surface receptors to second messengers to generate effector enzymes or ion channels [1,2]. G proteins are heterotrimers composed of three different subunits, α, β, and γ, and each of these subunits has been reported to exhibit molecular heterogeneity. To date, cDNAs encoding 21 distinct α subunits, four β subunits and six γ subunits have been cloned [2,3]. The β and γ subunits are believed to exist as a tightly associated complex that functions as a unit. The $\beta\gamma$ complexes promote interaction of the α subunits with receptors and regulate the rate of dissociation of guanine nucleotides from α subunits. In addition, the $\beta\gamma$ complexes interact directly or indirectly with various effectors, which include phospholipase A_2, cytosolic phospholipase C, adenylyl cyclase and potassium channels [2,3]. Functional differences among various forms of $\beta\gamma$ complexes seem to be attributable to the γ rather than to the β subunit [4,5]. Among several γ subunits, γ_1 and γ_3 are specifically expressed in rod outer segments and brain, respectively [2,3,5–8], while γ_2, γ_5, γ_7 and a novel form of γ, tentatively named γ_{S1}, are widely distributed in a variety of tissues [2,5–10]. Bovine brain mainly contains three forms of γ subunits, namely, γ_2, γ_3, and γ_7 [5–9,11,12].

It is generally accepted that the β and γ subunits can be separated only under denaturing conditions [1]. However, we found one form of the γ subunit, free of the corresponding β subunit, during purification of $\beta\gamma$ complexes from bovine brain under non-denaturing conditions . We report here the isolation of the γ subunit free from the β subunit. The isolated γ subunit was identified as γ_3.

2. Materials and methods

2.1. Isolation of subunits of G protein

The γ subunit, free of the corresponding β subunit, was isolated from bovine brain as follows. A cholate extract of membranes was subjected to successive column-chromatographic fractionations by the method of Asano et al. [5]. During the initial chromatography on DEAE-Sephacel, the fractions containing $\beta\gamma$ complexes were partially separated from the main fractions (Fraction I) that contained GTPγS-binding activities, and the former fractions were designated Fraction II, as described in a previous paper [5]. Fractions I and II contained relatively large amounts of γ_2 and γ_7, respectively, but both contained similar amount of γ_3 [5]. Fraction II was applied to a column of Ultrogel AcA 34, and the fractions containing $\beta\gamma$ complexes were then applied to a column (1.5 × 25 cm) of heptylamine-Sepharose which had been equilibrated with 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 5 mM β-mercaptoethanol, 20 μM $AlCl_3$, 6 mM $MgCl_2$, and 10 mM NaF (TES/AMF), supplemented with 100 mM NaCl and 0.25% sodium cholate. The column was washed with TES/AMF that contained 0.25% sodium cholate and 300 mM NaCl. Then, $\beta\gamma$ complexes were eluted with a 400-ml linear gradient of 0.2–0.05 M NaCl/0.25–1% sodium cholate in TES/AMF (Fig. 1). Fractions 54–60 were pooled and concentrated by ultrafiltration with a membrane (UP 20, Advantec Toyo, Tokyo, Japan). The concentrated fractions were diluted with 5 volumes of 20 mM Tris-HCl (pH 8.0), 0.1 mM EDTA and 0.7% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and then applied to Mono-Q HR 5/5 column (Pharmacia LKB, Uppsala, Sweden) which had been equilibrated with 20 mM Tris-HCl (pH 8.0), 0.1 mM EDTA and 0.7% CHAPS. The column was washed with the equilibration buffer and the protein was

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eluted with the linear gradient of NaCl (0–0.3 M, 30 ml; and 0.3–1 M, 5 ml) in the same buffer at a flow rate of 1 ml/min in an FPLC system from Pharmacia LKB (Fig. 2). Each fraction (1 ml) was collected in a tube that contained 10 μl of 100 mM dithiothreitol. Fractions 10 and 11 were pooled and stored as the free form of the γ subunit.

The βγ complex that contained only γ_3 ($\beta\gamma_3$) and trimeric G_o were purified from bovine brain by the method of Asano et al. [5] and by the method of Katada et al. [13], respectively.

2.2. Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli [14] with 10% polyacrylamide gels. For the analysis of γ subunits, Tricine/SDS-PAGE was performed by the method of Schägger and von Jagow [15] with a separating gel (16.5% polyacrylamide) that contained 13.3% glycerol. As sources of standards, low-molecular mass and polypeptide-molecular mass electrophoresis calibration kits (Pharmacia LKB) were used for SDS-PAGE and Tricine/SDS-PAGE, respectively.

2.3. Amino acid sequence analysis

The isolated γ subunit (15 μg) and the $\beta\gamma_3$ complex (15 μg) were subjected directly to amino acid sequencing on a gas-phase automated sequencer (PSQ-1; Shimazu, Kyoto, Japan).

2.4. Other methods

The immunoassay specific for the βγ complex was carried out by the method of Asano et al. [16]. Proteins were quantitated by the method of Schaffner and Weissmann with bovine serum albumin as the standard [17].

3. Results and discussion

Fig. 1 shows the elution profiles of βγ complexes from the heptylamine-Sepharose column. Analysis by SDS-PAGE (Fig. 1B) revealed that fractions mainly contained β and γ subunits. Analysis by Tricine/SDS-PAGE (Fig. 1C) showed that fractions contained three forms of γ subunits. γ_3 generated two bands during Tricine/SDS-PAGE. Because both bands reacted with the antibody against γ_3, they seemed to be γ_3 [5], but the reason for two bands was not clear. βγ complexes containing γ_3 were partially separated from those that contained γ_7, and γ_3-rich fractions were subjected to chromatography on a Mono-Q column. As shown in Fig. 2A, three peaks were detected by monitoring absorbance at 280 nm. Electrophoretic analysis showed that the major peak contained βγ complexes composed of different γ subunits (Fig. 2B and C). The same electrophoretic analyses showed that the minor peak, which had eluted earlier than the major peak, contained only one form of γ subunit which was free of the β subunit. Another minor peak, eluted by a high concentration of NaCl (fraction 34), contained the β subunit, which was identified with the antibody against the β subunit ([16]; data not shown), but it did not contain a significant amount of the γ subunit (Fig. 2B and C). When βγ complexes in the major peak fraction were rechromatographed on a Mono-Q column, no further free form of the γ subunit was observed. Fig. 3 shows results of SDS-PAGE and Tricine/SDS-PAGE of the final preparation of the γ subunit that had been freed from the β subunit. The preparation contained no α or β subunits, and it contained

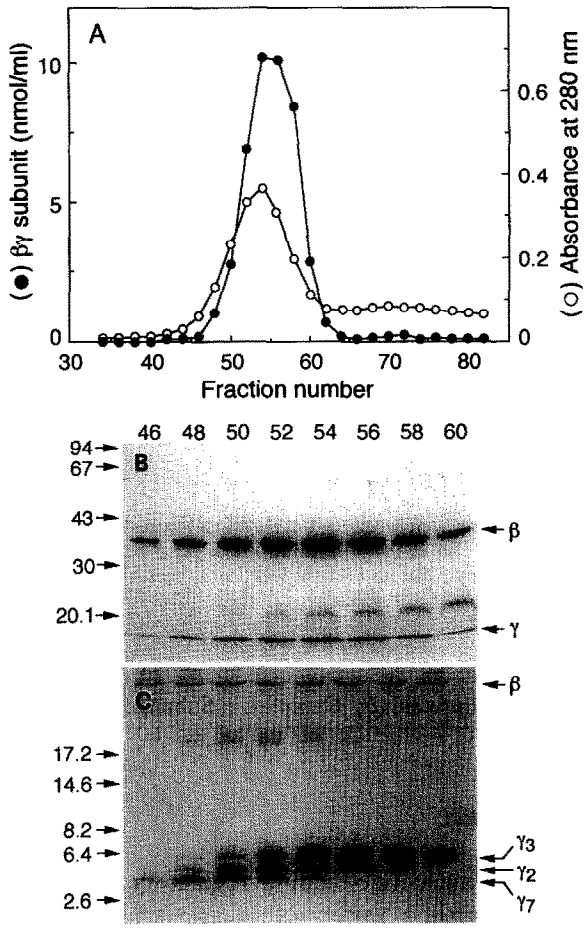

Fig. 1. Column chromatography on heptylamine-Sepharose of βγ complexes from bovine brain. (A) The βγ complex-rich fractions from Ultrogel AcA 34 were applied to a column of heptylamine-Sepharose and were eluted as described in section 2. Closed circles show concentrations of βγ complexes, as quantitated by immunoassay with a mixture of βγ complexes from brain as the standard. Open circles show absorbance at 280 nm. (B) Patterns after SDS-PAGE and staining with Coomassie blue of the column fractions. (C) Patterns after Tricine/SDS-PAGE and silver staining of the column fractions. Numbers on the left indicate molecular masses in kDa. Fractions 54–60 were pooled for further purification.

only one form of γ subunit which had identical mobility to γ_3. Approximately 50 μg of γ subunit were obtained from 500 g of bovine brain.

To determine the primary structure of the isolated γ subunit, the γ subunit was subjected to amino acid sequence analysis. The amino acid sequence obtained from the free form of the γ subunit (MKGETPVNSTM-SIGQARKM) was identical to the N-terminal sequence of γ_3 ([6]; Met^1–Met^{19}). Because the N-termini of most γ subunits seem to be modified [10–12], it appeared possible that N-terminal modification was absent only in the free form of γ_3. To examine whether the N-terminus of γ_3 that was associated with the β subunit ($\beta\gamma_3$) was blocked, the $\beta\gamma_3$ complex was directly subjected to Edman degradation. Methionine was established as the

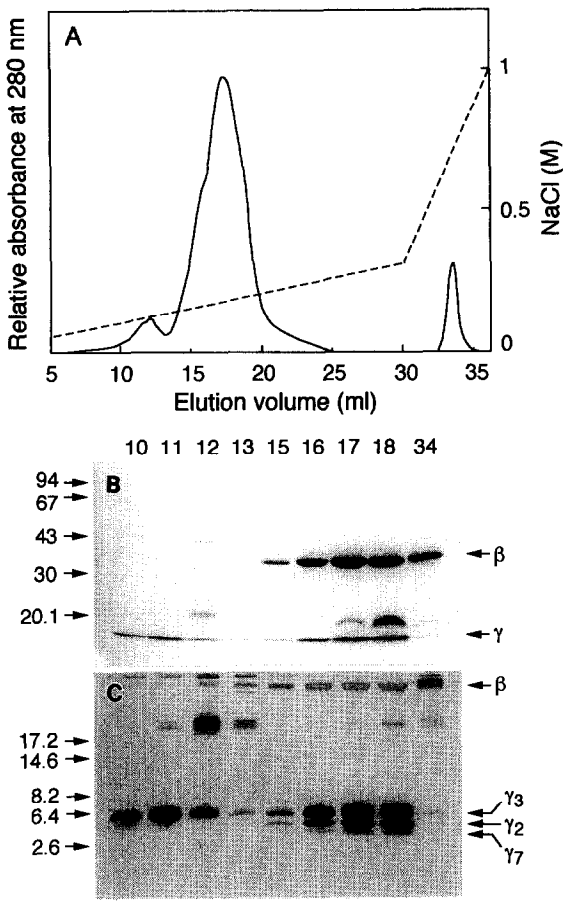

Fig. 2. Chromatography on a Mono-Q column of $\beta\gamma$ complexes from bovine brain. (A) The $\beta\gamma$ complex-containing fractions from heptylamine-Sepharose were applied to the Mono-Q column and were eluted as described in section 2. The solid line shows absorbance at 280 nm. The broken line shows the concentration of NaCl. (B) Patterns after SDS- PAGE and staining with Coomassie blue of the column fractions. (C) Patterns after Tricine/SDS-PAGE and silver staining of the column fractions. Numbers on the left indicate molecular masses in kDa.

N-terminal residue and continuous analysis revealed the N-terminal sequence of γ_3 ([6]; MKGETPVNSTM-SIGQARKM). The molar recovery of N-terminal methionine represented approximately 60% of the $\beta\gamma_3$ complex applied to the protein sequencer, indicating that the N-terminal methionine of γ_3 that was associated with the β subunit was not modified and resembled that of the free form of γ_3.

To determine whether γ_3, free of the β subunit, had biological activities similar to those of the $\beta\gamma$ complex, the isolated γ_3 was examined in various experiments. However, the free form of γ_3 was not effective in facilitating ADP-ribosylation of the α subunit of G_o by pertussis toxin or in inhibiting calmodulin-stimulated adenylyl cyclase activity. In addition, sucrose density gradient centrifugation showed that γ_3 did not associate with the α subunit of either G_o or G_{i1} (data not shown).

As mentioned above, the $\beta\gamma$ complexes were divided between two fractions (Fractions I and II) during the first step in the purification [5] and the free form of γ_3 was isolated from Fraction II in the present study. When $\beta\gamma$ complexes were purified from Fraction I, which mainly contained γ_2 and γ_3 [5], by a similar method, γ_3 free of the β subunit was also obtained. Thus, considerable amounts of the free form of γ_3 could be purified from bovine brain. The results shown in Fig. 2 suggest that the free form of γ_3 was derived from a $\beta\gamma_3$ complex. At the present time, it is not clear whether γ_3 was freed from the β subunit in the cells or during purification. However, it is unlikely that the dissociation is simply due to the instability of the $\beta\gamma_3$ complex, because the free form of γ_3 was not obtained from purified $\beta\gamma_3$ complex that was stored at 4°C for 10 days.

In contrast to relatively large amounts of free γ_3, significant amounts of the free forms of the other γ subunits in the brain (γ_2 and γ_7) were not obtained from any fractions examined. To date, six forms of γ subunits have been purified from various bovine tissues and subjected to Edman degradation. No PTH-derivatized amino acids have been detected in the analysis of γ_2 [11,12], γ_5 [10], γ_7 [10–12] and γ_{S1} ([5]; unpublished data), presumably because of modification of the N-termini of these proteins. Indeed, Sohma et al. [12] indicated that the N-termini of γ_2 and γ_7 seemed, respectively, to be acylated alanine and acylated serine residues at position 2. By contrast, the N-terminal residue of γ_3 was found to be unmodified methionine in the present study. In addition, the N-terminal residue of γ_1 was reported to be unmodified proline at position 2 [18]. Yamazaki et al. [19] isolated β subunits freed from γ_1 during purification of the $\beta\gamma$ complex from rod outer segments of *Bufo marinus*, though they did not isolate the free form of γ_1. These results suggest that modification at the N-terminus of γ subunits might increase the affinity of each for β subunits. With respect to the localization of γ subunits, both γ_1 and γ_3, whose N-termini were not modified, were found to be specifically expressed in rod outer segments and brain, respectively. By contrast, γ_2, γ_5, γ_7 and γ_{S1}, with modified N-termini, are widely distributed in a vari-

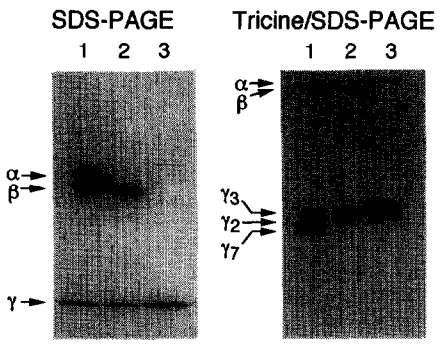

Fig. 3. Patterns after SDS-PAGE and Tricine/SDS-PAGE of trimeric G_o, $\beta\gamma_3$ and the isolated γ subunit. Samples were subjected to SDS-PAGE or Tricine/SDS-PAGE and proteins were visualized by staining with Coomassie blue (left) or silver (right). Lane 1, G_o (1 μg); lane 2, $\beta\gamma_3$ (0.5 μg); lane 3, the isolated γ subunit (0.2 μg).

ety of tissues [2,3,5–10]. The low affinity of γ_3 and γ_1 for the β subunit may reflect the specific functions of these γ subunits in nervous tissues and rod outer segments, respectively.

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References

[1] Neer, E.J. and Clapham, D.E. (1988) Nature 333, 129–134.
[2] Hepler, J.R. and Gilman, A.G. (1992) Trends Biochem. Sci. 17, 383–387.
[3] Birnbaumer, L. (1992) Cell 71,1069–1072.
[4] Iñiguez-Lluhi, J., Simon, M.I., Robishaw, J.D. and Gilman, A.G. (1992) J. Biol. Chem. 267, 23409–23417.
[5] Asano, T., Morishita, R., Matsuda, T., Fukada, Y., Yoshizawa, T. and Kato, K. (1993) J. Biol. Chem. 268, 20512–20519.
[6] Gautam, N., Northup, J., Tamir, H. and Simon, M.I. (1990) Proc. Natl. Acad. Sci. USA 87, 7973–7977.
[7] Cali, J.J., Balcueva, E.A., Rybalkin, I. and Robishaw, J.D. (1992) J. Biol. Chem. 267, 24023–24027.
[8] Morishita, R., Fukada, Y., Kokame, K., Yoshizawa, T., Masuda, K., Niwa, M., Kato, K. and Asano, T. (1992) Eur. J. Biochem. 210, 1061–1069.
[9] Gautam, N., Baetscher, M., Aebersold, R. and Simon, M.I. (1989) Science 244, 971–974.
[10] Morishita, R., Masuda, K., Niwa, M., Kato, K. and Asano, T. (1993) Biochem. Biophys. Res. Commun. 194, 1221–1227.
[11] Robishaw, J.D., Kalman, V.K., Moomaw, C.R. and Slaughter, C.A. (1989) J. Biol. Chem. 264, 15758–15761.
[12] Sohma, H., Hashimoto, H., Hiraike, N., Ohguro, H. and Akino, T. (1993) Biochem. Biophys. Res. Commun. 190, 849–856.
[13] Katada, T., Oinuma, M., Kusakabe, K. and Ui, M. (1987) FEBS Lett. 213, 353–358.
[14] Laemmli, U.K. (1970) Nature 227, 680–685.
[15] Schägger, H. and von Jagow, G. (1987) Anal. Biochem. 166, 368–379.
[16] Asano, T., Kamiya, N., Morishita, R. and Kato, K. (1988) J. Biochem. (Tokyo) 103, 950–953.
[17] Schaffner, W. and Weissmann, C. (1973) Anal. Biochem. 56, 502–514.
[18] Ovchinnikov, Yu.A., Lipkin, V.M., Shuvaeva, T.M., Bogachuk, A.P. and Shemyakin, V.V. (1985) FEBS Lett. 179, 107–110.
[19] Yamazaki, A., Tatsumi, M., Torney, D.C. and Bitensky, M.W. (1987) J. Biol. Chem. 262, 9316–9323.