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ISOLATION OF THREE TYPES OF G_i FROM BOVINE SPLEEN[1]

Rika Morishita, Tamio Kobayashi*, Kanefusa Kato and Tomiko Asano[2]

Department of Biochemistry, Institute for Developmental Research, Aichi Prefectural Colony, Kasugai, Aichi 480-03, Japan

* Medical and Biological Laboratories, Ina, Nagano 396, Japan

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SUMMARY: We have previously reported the purification of two α subunits of G proteins, G_{i2} and G_{i3}, from bovine spleen. However, it recently became clear that the preparation of $G_{i3}\alpha$ contained a significant amount of $G_{i1}\alpha$ by the immunoblot analysis using specific antibodies. In this study, we purified these G proteins as a trimer form from bovine spleen, and obtained following results. (1) G_{i3} was separated from G_{i1} using Mono Q column chromatography. Isoelectric focusing was employed to distinguish G_{i3} from G_{i1} in the column eluates. (2) Purified G_{i2} and G_{i3} retained much higher activities to bind GTPγS or to be ADP-ribosylated by pertussis toxin than the α subunits purified previously. (3) Using these spleen G_{i2} and G_{i3} and bovine brain G_{i1}, the parameter of GTPγS binding to the three types of G_i was compared. Three G_is showed different rates of GTPγS binding but showed the similar Kd values.

G proteins couple cell surface receptors to a variety of effectors including enzymes and ion channels serving as signal transducers (1). G proteins are a family of heterotrimeric proteins (α, β and γ subunits) and structurally homologous. Among G proteins, G_i, G_o and transducin are known to be substrates for ADP-ribosylation catalyzed by pertussis toxin (IAP). Molecular cloning indicated the existence of at least three types of G_i termed G_{i1}, G_{i2} and G_{i3} (1). The α subunits of these G_is are significantly more similar to each other than the α subunits of G_o and transducin. $G_{i1}\alpha$ and $G_{i3}\alpha$ are 94% identical in their amino acid sequence, and they are 88 and 85% identical, respectively, to $G_{i2}\alpha$ (2,3).

There are many reports on identification of 41-kDa α and 40-kDa α subunits purified from various tissues as the proteins encoded by $G_{i1}\alpha/G_{i3}\alpha$ and $G_{i2}\alpha$ cDNAs (4-10). Among the three types of G_i, only G_{i3} have not been obtained yet as a pure and active form (10,11). It is because the tissue which contained a large amount of G_{i3} was not found and the separation of G_{i3} from G_{i1} was not successful due to their similarity. We have previously purified two α subunits of IAP-sensitive G proteins with molecular masses of 41 kDa and 40 kDa from bovine spleen and identified as $G_{i3}\alpha$ and $G_{i2}\alpha$, respectively (10). At that time we were not able to find $G_{i1}\alpha$ in the $G_{i3}\alpha$ preparations, because $G_{i1}\alpha$ and $G_{i3}\alpha$ displayed the identical

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[2] To whom requests for reprints should be addressed.

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molecular weight on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the analysis of partial amino acid sequence did not detected the tryptic peptides of $G_{i1}\alpha$. However, we have recently found that $G_{i3}\alpha$ preparations purified from bovine spleen contained a significant amount of $G_{i1}\alpha$ by immunoblot analysis with antibodies specific to $G_{i1}\alpha$ (12). In addition, purified α subunits of G_{i2} and G_{i3} retained relatively low activities of GTPγS binding and ADP-ribosylation by IAP (10). In this study, we purified G_is as a trimer form from bovine spleen, and separated G_{i3} from G_{i1}. Because purified G_{i2} and G_{i3} retained a high activity of GTPγS binding, the kinetics of GTPγS binding to the three types of G_i were studied.

METHODS

Purification of G proteins——Membranes were prepared from about 1kg of bovine spleen, and DEAE-Sephacel and Ultrogel AcA-34 column chromatographies were performed as described previously (10). The fractions containing IAP-sensitive G proteins were then applied to a heptylamine-Sepharose column. The chromatography was carried out as described previously (10) except that in the buffer without $AlCl_3$, $MgCl_2$ and NaF to obtain G proteins as a trimer form. The peak fractions from the heptylamine-Sepharose column were pooled and concentrated to 8 ml by ultrafiltration with membrane (Toyo UP-20). The concentrated fraction was diluted with 4 volumes of 20 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 5 mM 2-mercaptoethanol (TES) and 0.5% Lubrol PX, and was applied to a column (1.5 x 20 cm) of DEAE-Toyopearl 650(S) which had been equilibrated with TES/0.5% Lubrol PX/25 mM NaCl. The column was washed with the equilibration buffer and eluted with a 400 ml linear gradient of 25-250 mM NaCl in TES/0.5% Lubrol PX. The elution profiles of the activities from the column and SDS-PAGE of each fraction are shown in Fig. 1. This chromatography resulted in a separation of two peaks of IAP-sensitive G proteins. The minor and major peaks contained G proteins with α subunits of 41 kDa and 40 kDa, respectively. The fractions 22-25 of minor peak were pooled and diluted with 2 volumes of 20 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA (TE) and then applied to a Mono Q HR 5/5 column which had been equilibrated with TE/0.7% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Chaps). The column was washed with 125 mM NaCl in TE/0.7% Chaps and eluted at a flow rate of 1 ml/min with a 30 ml linear gradient of 125-300 mM NaCl in the same buffer using a Pharmacia FPLC system. Each fraction (1 ml) was collected in a tube which contained 10 µl of 100 mM dithiothreitol. The elution profiles of the activities from the column are shown in Fig. 2A. Each fraction containing the activities was subjected to isoelectric focusing, and immunoblot analysis was performed as described below (Fig. 2B). Fraction 12 was stored as spleen G_{i1} fraction at -80 °C. The fractions 14-16 were pooled and rechromatographed on the same column, and the final preparation was stored as spleen G_{i3} fraction.

The fractions 27-31 of major peak containing G_{i2} eluted from DEAE-Toyopearl 650(S) column chromatography were also pooled and purified by Mono Q column chromatography under the same conditions as described above.

Bovine brain G_{i1} was purified by the method of Katada et al. (13). The α subunit of G_{i3} was purified from bovine spleen by the method described previously (10). Proteins were determined by the method of Schaffner and Weissman (14).

Assay of activities——ADP-ribosylation catalyzed by IAP was performed essentially by the method of Bokoch et al. (15). Binding of [^{35}S]GTPγS was measured by the method described by Northup et al. (16).

Electrophoresis and Immunoblotting——SDS-PAGE was carried out by the method of Laemmli (17). Isoelectric focusing gels were prepared essentially as described by O'Farrell (18) except that in glass plates instead of glass tubes. Purified G proteins or aliquots of fractions was added to 4 volumes of a solution containing 9.5 M urea, 2% ampholines (LKB, 1.6% 5/7 and 0.4% 3/10), 2% Nonidet P-40, and 5% 2-mercaptoethanol, and were then applied to the basic end of the isoelectric focusing gels. Isoelectric focusing was performed at 400 V for 16 h in a slab gel apparatus (Bio-Rad) using 50 mM NaOH as the catholyte and 30 mM H_3PO_4 as the anolyte. Proteins were electrophoretically transferred from SDS-polyacrylamide gels or isoelectric focusing gels to nitrocellulose or Immobilon (Millipore) sheets, respectively, by the method of Towbin et al. (19). Immunoblotting was then

performed as described previously (20) with 0.05 μg/ml of antibodies or 1:500 dilution of antiserum.

The purification and characterization of antibodies to $G_{i1}\alpha$, $G_{i2}\alpha$ or $G_{i1}\alpha/G_{i3}\alpha$ were described previously (12,20). Anti-$G_{i1}\alpha$ and anti-$G_{i1}\alpha/G_{i3}\alpha$ antibodies were referred to as anti-$G_{i1}\alpha$ peptide and anti-$G_{i1}\alpha$ protein antibodies, respectively, in our previous paper (12). The antiserum against $G_{i3}\alpha$ was kindly provided by Dr. Y. Kanaho (Gifu University) (21).

RESULTS

After successive column chromatographies on DEAE-Sephacel, Ultrogel AcA-34 and heptylamine-Sepharose, IAP-sensitive G proteins were subjected to DEAE-Toyopearl 650(S) column chromatography. On this chromatography, the minor G proteins with 41-kDa α subunits were separated from the major G protein with 40-kDa α subunit (Fig. 1). The major G protein was identified as G_{i2} in our previous paper (10). The fractions containing a minor peak were then subjected to Mono Q column chromatography, and eluates were analyzed by isoelectric focusing, followed by immunoblotting with anti-$G_{i1}\alpha/G_{i3}\alpha$ antibodies (12). As shown in Fig. 2B, $G_{i1}\alpha$ and $G_{i3}\alpha$ showed clearly different isoelectric points, and G_{i1} was eluted earlier than G_{i3} on this chromatography. The isoelectric points of α subunits of G_{i1} and G_{i3} were 6.2 and 6.0, respectively, in agreement with the results obtained with bovine brain $G_{i1}\alpha$ and a translation product encoded by $G_{i3}\alpha$ cDNA (22). Thus, bovine spleen contained G_{i1} in addition to G_{i2} and G_{i3} which were identified previously (10). The order of the contents of three types of G_i was $G_{i2}>>G_{i3}>G_{i1}$. As shown in Fig. 3A, SDS-PAGE analysis of the final preparations revealed that both G_{i2} and G_{i3} preparations were almost homogeneous, while G_{i1} preparations contained a small amount of a contaminant with a 57-kDa molecular mass. To confirm our present identification of the three purified G_is, immunoblot analyses using various antibodies or antiserum were carried out (Fig. 3B, C, D). Anti-$G_{i1}\alpha$ antibodies (12) reacted with both α subunits of brain and spleen G_{i1}, but not with

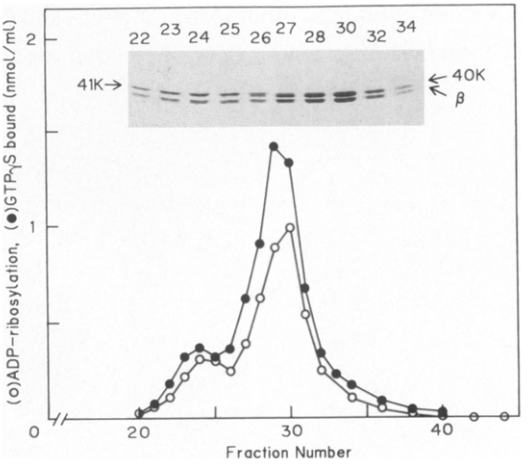

Fig. 1. **DEAE-Toyopearl 650(S) column chromatography of IAP-sensitive G proteins.** The fractions containing IAP-sensitive G proteins from heptylamine-Sepharose column were applied to a DEAE-Toyopearl 650(S) column and eluted as described in "METHODS". Aliquots (10 μl) of fractions were assayed for ADP-ribosylation by IAP (○) and [^{35}S]GTPγS binding (●) activities. The inset shows the Coomassie blue stain of protein analyzed by SDS-PAGE. The number of each lane corresponds to the fraction number.

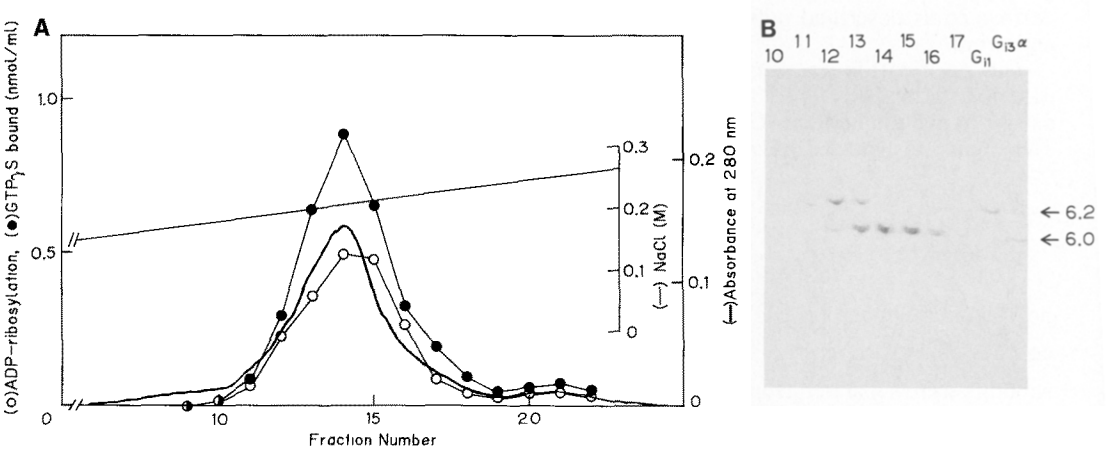

Fig. 2. **Mono Q column chromatography of G proteins with 41-kDa α subunits.** (A) The minor peak fraction of DEAE-Toyopearl 650(S) column was applied to a Mono Q column and eluted as described in "METHODS". Aliquots (10 μl) of fractions were assayed for ADP-ribosylation by IAP (o) and [^{35}S]GTPγS binding (●) activities. The absorbance at 280 nm of the eluted protein was also monitored (—). (B) Purified brain G_{i1} (0.4 μg), spleen $G_{i3}\alpha$ (0.2 μg) and aliquots (3 μl) of fractions were subjected to isoelectric focusing and analyzed by immunoblotting with anti-$G_{i1}\alpha$/$G_{i3}\alpha$ antibodies. The number of each lane corresponds to the fraction number. The approximate isoelectric point range is from 7.5 to 4.0 (top to bottom). Numbers on the right indicate isoelectric points.

$G_{i2}\alpha$ or $G_{i3}\alpha$ (Fig. 3B). Anti-$G_{i2}\alpha$ antibodies (20) reacted only with $G_{i2}\alpha$ (Fig. 3C). The $G_{i3}\alpha$ antiserum (21) reacted strongly with the $G_{i3}\alpha$, and weakly with the α subunits of spleen G_{i1} or G_{i2} (Fig. 3D), suggesting that the spleen G_{i1} and G_{i2} preparations were contaminated with a small amount of G_{i3}.

The maximal binding of GTPγS to both purified G_{i2} and G_{i3} were from 0.5 to 0.6 mol/mol protein (Table 1), and the maximal incorporations of [^{3}H]ADP-ribose into both G_{i2} and G_{i3} by IAP were from 0.4 to 0.5 mol/mol protein. Both activities of G_{i2} and G_{i3} purified as a trimer form were higher than those of $G_{i2}\alpha$ and $G_{i3}\alpha$ purified as a monomer form described previously (10).

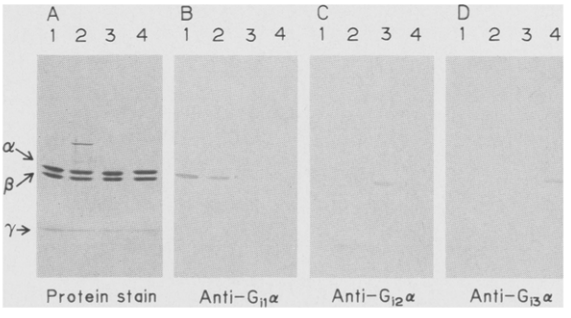

Fig. 3. **SDS-PAGE and immunoblot of the three types of G_i purified from spleen and brain G_{i1}.** Proteins (1 μg) were subjected to SDS-PAGE. The gel was then stained with Coomassie blue (A) or immunoblotted with anti-$G_{i1}\alpha$ peptide (B) or anti-$G_{i2}\alpha$ antibodies (C) or $G_{i3}\alpha$ antiserum (D). Lane 1, brain G_{i1}; lane 2, spleen G_{i1}; lane 3, spleen G_{i2}; lane 4, spleen G_{i3}.

TABLE 1. *The Kd values for GTPγS of the three types of G_i*

	Kd (nM)	Bmax (mol/mol of protein)
G_{i1}	11	0.72
G_{i2}	14	0.53
G_{i3}	14	0.54

Brain G_{i1}, spleen G_{i2} or G_{i3} (1 pmol) was incubated at 30 °C for 1 h in 200 μl of 20 mM Na-Hepes (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, 0.1% Lubrol PX, 1 mg/ml bovine serum albumin, 30 mM $MgCl_2$ and various concentrations of [^{35}S]GTPγS. The values of Kd and Bmax were obtained from Scatchard plots. Data are mean values from three experiments.

To compare properties of the three types of G_i, the kinetics of GTPγS binding was studied in the presence of various concentrations of Mg^{2+}. Bovine brain G_{i1} was used instead of spleen G_{i1}, because the G_{i1} purified from the spleen was not sufficient for the above studies. The rates of GTPγS binding to any G_i displayed apparent first-order kinetics over a wide range of Mg^{2+} concentrations (not shown). The apparent first-order rate constant (k_{app}) of three types of G_i obtained from first-order plots increased with increasing concentrations of Mg^{2+} (Fig. 4). The k_{app} of G_{i2} was the largest among three G_is at low concentrations of Mg^{2+}, but the rate of GTPγS binding to G_{i1} was greatly increased at higher concentrations of Mg^{2+} more than 30 mM (Fig. 4). G_{i3} always showed the smallest k_{app}. Kd values for GTPγS of G_{i1}, G_{i2} and G_{i3} were obtained by Scatchard plots and were shown in Table 1. Kd values of the three G_is were almost same and also similar to those of rabbit liver G_i (15) or bovine brain $G_o\alpha$ (23).

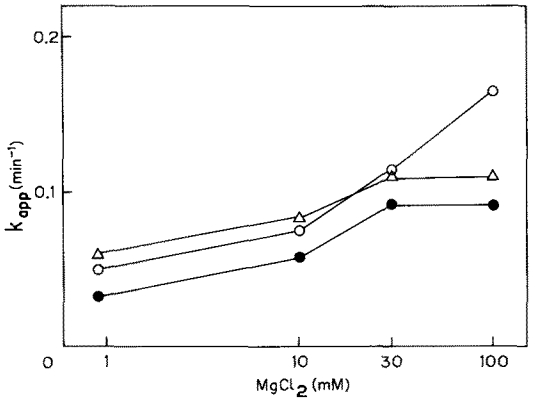

Fig. 4. **Kinetics for GTPγS binding to the three types of G_i at various concentrations of $MgCl_2$.** Brain G_{i1}, spleen G_{i2} and G_{i3} (4 μg) were incubated at 30 °C in 400 μl of 20 mM Na-Hepes (pH 8.0), 0.1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, 0.1% Lubrol PX, 1 mg/ml bovine serum albumin, 1 μM [^{35}S]GTPγS and various indicated concentrations of $MgCl_2$. At various times, aliquots (50 μl) were withdrawn and assayed for [^{35}S]GTPγS binding. The amounts of GTPγS binding were shown as first-order plots (not shown). The plateau levels of bound GTPγS were averaged for each concentration of $MgCl_2$ to yield B_T. The values of B_T were not significantly altered by changing concentrations of $MgCl_2$; B_T values of 2.8-3.2, 2.3-2.5 and 2.6-2.7 pmol/assay were obtained for brain G_{i1}, spleen G_{i2} and G_{i3}, respectively, under the conditions tested. The apparent first-order rate constants (k_{app}) were determined from first-order plots, and plotted against concentrations of $MgCl_2$ used. G proteins were as follows; brain G_{i1} (○), spleen G_{i2} (△) and spleen G_{i3} (●).

DISCUSSION

Three G_i molecules were isolated from each other by successive DEAE chromatographies. A mixture of G_{i1} and G_{i3} were separated from G_{i2} on the DEAE-Toyopearl 650(S) chromatography, which brought a better separation as compared with the DEAE-Sephacel chromatography used for the purification of α subunits (10). Then G_{i3} was separated from G_{i1} by Mono Q column chromatography. SDS-PAGE was not applicable to recognize G_{i1} and G_{i3} in the column eluates because of their apparently identical molecular weights. Then, isoelectric focusing was performed, because the α subunits of G_{i1} and G_{i3} had different isoelectric points (22). The α subunits were detected with antibodies reactive with both G_{i1} and G_{i3}. The α subunits of G_{i1} and G_{i3} were clearly distinguished from each other on the gels. The present study revealed that bovine spleen contained three types of G_i in order of the contents of G_{i2}>>G_{i3}>G_{i1}. This result is consistent with the fact that all three $G_i\alpha$ mRNAs were expressed in human spleen (24).

When we purified the α subunits of G_{i2} and G_{i3} from bovine spleen, both α subunits showed low activities of both GTPγS binding and ADP-ribosylation by IAP even in the presence of $\beta\gamma$ (10). However, in the present study, G_{i2} and G_{i3} were purified as a trimer form and showed much higher activities than $G_{i2}\alpha$ and $G_{i3}\alpha$ purified previously (10). These results suggest that $\beta\gamma$ subunits protected the inactivation of α subunits during the purification.

Because we obtained the sufficiently active G_{i3}, we compared the parameters of GTPγS binding to the three types of G_i. The Kd values for GTPγS of the three types of G_i were approximately same. However, the rates of GTPγS binding to these G proteins were significantly different. The order of the rates of GTPγS binding was G_{i2}>G_{i1}>G_{i3} at lower concentrations of Mg^{2+} (<10 mM), but that was G_{i1}>G_{i2}>G_{i3} at higher concentrations of Mg^{2+} (>30 mM). Linder et al. (25) recently reported that the rates of GTPγS binding to recombinant $G_{i1}\alpha$ and $G_{i3}\alpha$ were similar, and was 2-3 times slower than that to recombinant $G_{i2}\alpha$ in the presence of 10 mM Mg^{2+}, showing the data similar to the present results. Because $G_{i1}\alpha$ and $G_{i3}\alpha$ are 94% homologous in their amino acid sequence, the rates of GTPγS binding to them were expected to be similar. Difference of the rates at high concentrations of Mg^{2+} might be caused by the $\beta\gamma$ subunits.

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