Identification of two forms of the γ subunit of G protein, γ_{10} and γ_{11}, in bovine lung and their tissue distribution in the rat

Rika Morishita, Hiroshi Ueda, Kanefusa Kato, Tomiko Asano*

Department of Biochemistry, Institute for Developmental Research, Aichi Human Service Center, Kamiya, Kasugai, Aichi 480-0392, Japan

Received 13 March 1998; accepted 20 April 1998

Abstract Two forms of the γ subunit of G protein were purified from bovine lung, and were identified as γ_{10} and γ_{11} by analyses of partial amino acid sequences and reactivity with specific antibodies. The N-terminal amino acid residue of γ_{11} was an unmodified Pro^2, and the purified γ_{11} was freed from β even under non-denaturing conditions. Western blots with specific antibodies against γ_{10} and γ_{11} showed that both γ subunits are present in a variety of tissues in the rat, with a particular abundance of γ_{11} in the platelets.

Key words: G protein γ subunit; Purification; Antibody; Localization

1. Introduction

Intracellular transmission of extracellular signals is most commonly mediated by a family of G proteins that couple with various receptors and effectors to produce appropriate cellular responses [1]. G proteins consist of three subunits, α, β and γ, and the latter two exist as a tightly bound complex. The α and βγ complex of G proteins are maintained in an inactive state by their mutual association in a heterotrimeric complex. Upon activation, both the GTP-bound α subunit and the released βγ complexes are free to interact with downstream components of the signalling cascade. At present, complete cDNAs encoding five β subunits and eleven γ subunits have been cloned from mammals [2,3]. At the amino acid level, the β subunits show high identity, whereas the γ subunits are more divergent. Functional differences among various forms of βγ complexes have been attributed to the γ rather than to the β subunit [4–6]. In particular, the biological properties of $\beta\gamma_1$ are noticeably different from those of the other βγ complexes. Among five isoforms of the β subunits (β_1–β_5), β_1–β_4 subunits are expressed ubiquitously, whereas β_5 subunit is expressed only in the brain [2]. By contrast, γ subunits show more variation in their tissue specific distribution. The γ_1 subunit and γ_c are specifically expressed in retinal rods and cones, respectively [7–9]. The γ_8 is expressed only in olfactory and vomeronasal neuroepithelia [10], whereas γ_3 and γ_4 are localized only in the brain [11–16]. By contrast, γ_2, γ_5, γ_7 and γ_{12} are distributed in a variety of tissues [3,11–14]. It is likely that such differences in distribution are important in limiting the number of combinatorial associations of the α, β and γ subunits and consequently, functionally distinct G proteins.

To date, mRNAs of γ_{10} and γ_{11} have been detected in several tissues [15], but identification of γ_{10} and γ_{11} proteins in tissues has not been done. In amino acid sequence, γ_{11} is most similar to γ_1 [7,8,15]. In addition, γ_{11} is modified by a farnesyl group like γ_1 and γ_c which are expressed only in the retinae [9,15,17], while the other γ subunits are modified by a geranylgeranyl group [3,13,15,18,19]. Therefore, it is worth characterizing the γ_{11} protein and clarifying whether γ_{11} is abundant in specific tissues. In the present study, we isolated two forms of γ subunit from bovine lung and identified them as γ_{10} and γ_{11}. We also determined the tissue distribution of these two γ subunits in the rat with specific antibodies.

2. Materials and methods

2.1. Preparation of antibodies

Two peptides, *N*-acetyl-SSGASASALQRC and MPALHIEDLPEKEKC, corresponding to residues Ser^2-Arg^{12} of γ_{10} and Met^1-Lys^{14} of γ_{11}, respectively, appended with cysteine for coupling purposes, were synthesized. Antisera against γ_{10} and γ_{11} were raised in rabbits by the injection of each peptide conjugated to keyhole limpet hemocyanin. The antibodies were purified from antisera by the use of a column of Sepharose to which γ_{10} or γ_{11} peptide had been covalently coupled. Antibodies against β, γ_2 and γ_{12} have been described previously and characterized [3,13,14].

2.2. Separation of γ_{10} and γ_{11}

Two forms of γ subunits, γ_{10} and γ_{11}, were purified by successive column chromatography from bovine lung as described previously [5] with a modification. At each step after chromatography, fractions were subjected to Tricine/SDS-PAGE and immunoblotting for analyses of γ subunits. In brief, cholate extracts of membranes from bovine lung were applied to a column of DEAE-Sephacel, and the fractions containing βγ were divided into Fractions I and II as described in a previous paper for bovine brain [5]. Fraction I and II contained relatively large amounts of the immunoreactive γ_{11} and γ_{10}, respectively. Fraction I was subjected to successive column chromatography of Ultrogel AcA 34 and heptylamine-Sepharose. Then fractions containing the immunoreactive γ_{11} were applied to a Mono Q HR 5/5 column (Pharmacia Biotech, Tokyo, Japan) as described [20], and the immunoreactive γ_{11} was passed through this column (Fig. 1). The flow through fractions were concentrated by ultrafiltration with a membrane (UK 10, Advantec Tokyo, Tokyo, Japan), and then applied to a reversed-phase Cosmosil $5C_8$ column (0.46×15 cm, Nacalai Tesque, Kyoto, Japan). Proteins were eluted with a linear gradient of 5–90% acetonitrile (1%/min) in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. The peak fractions containing the immunoreactive γ_{11} were then applied to a reversed-phase Cosmosil $5C_{18}$-P300 column (0.46×15 cm, Nacalai Tesque, Kyoto, Japan), and the immunoreactive γ_{11} was eluted with a linear gradient of 5–90% acetonitrile (1%/min) in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min (Fig. 2).

Fraction II, which contained the immunoreactive γ_{10}, was subjected to successive column chromatography of Ultrogel AcA 34, heptylamine-Sepharose, and TSKgel phenyl-5PW (0.8×7.5 cm, Tosoh, Tokyo, Japan) as described previously [5]. Final preparation was eluted from a reversed-phase Cosmosil 5PE (0.8×25 cm, Nacalai Tesque, Kyoto, Japan) as described [13] (Fig. 3).

*Corresponding author. Fax: +81 (568) 88 0829.
E-mail: toasano@inst-hsc.pref.aichi.jp

Abbreviations: G proteins, heterotrimeric guanine nucleotide-binding regulatory proteins; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography

PII S0014-5793(98)00498-0

2.3. Amino acid sequence analysis

Approximately 10 μg of purified γ_{10} subunit fraction was digested with *S. aureus* V8 protease, and fragments separated by a reversed-phase HPLC were analyzed with Edman degradation as described previously [21]. The isolated γ_{11} subunit was subjected directly to amino acid sequencing.

2.4. Other methods

Tricine/SDS-PAGE was performed by the method of Schägger and von Jagow [22]. Immunoblotting was performed as described [14] by the use of chemiluminescence reagent (Renaissance, DuPont NEN). Cholate extracts of various rat tissues were prepared as described [14] and proteins were quantitated with a Micro bicinchoninic acid protein assay kit (Pierce) using bovine serum albumin as the standard.

3. Results

Two forms of γ subunit of G proteins were purified from bovine lung by successive column chromatography. When the cholate extract of membranes from bovine lung was subjected to DEAE-Sephacel column chromatography, fractions containing trimer forms of G protein (Fraction I) were partially separated from fractions that contained βγ complexes alone (Fraction II) as well as the extract from bovine brain [5] (data not shown). Most of the immunoreactive γ_{11} and γ_{10} were eluted in Fraction I and II, respectively.

Fraction I was then applied to a column of Ultrogel AcA 34. Analyses of fractions with various antibodies showed that the immunoreactive γ_{11} was eluted later than βγ complexes, suggesting most of the immunoreactive γ_{11} was freed from β subunit (data not shown). Fractions containing immunoreactive γ_{11} were then applied to a column of heptylamine-Sepharose and subsequently to a Mono Q column, which was previously used to isolate the γ_3 free from β subunits [20]. The flow through fraction contained the free immunoreactive γ_{11}

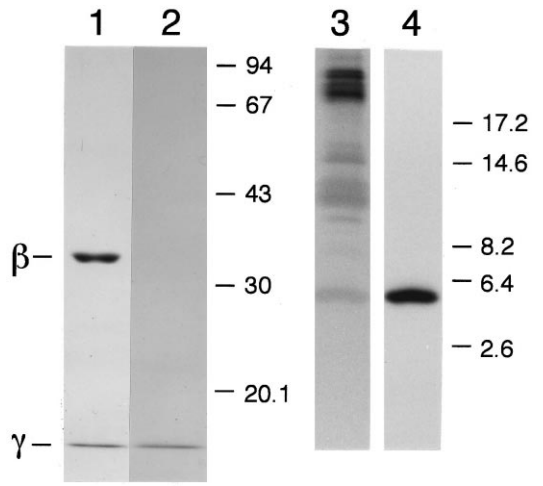

Fig. 1. Analyses of the flow-through fraction of Mono Q column chromatography of the immunoreactive γ_{11}. Fractions containing the immunoreactive γ_{11} from heptylamine-Sepharose were applied to a column of Mono Q. The column was washed with the buffer containing 20 mM Tris-HCl (pH 8.0), 0.1 mM EDTA and 0.7% CHAPS and then proteins were eluted with a linear gradient of NaCl. Since the immunoreactive γ_{11} was recovered in the flow-through fraction, it was analyzed by SDS-PAGE (lanes 1 and 2) or Tricine/SDS-PAGE (lanes 3 and 4). Lane 1: purified βγ stained with Coomassie blue; lane 2: flow-through fraction stained with Coomassie blue; lane 3: flow-through fraction stained with silver; lane 4: flow-through fraction immunoblotted with antibodies against γ_{11}. Numbers on the right indicate molecular masses in kilodaltons.

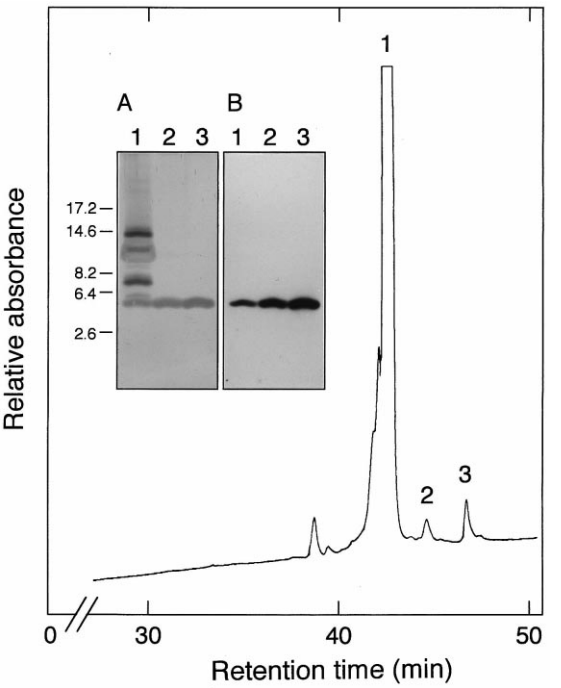

Fig. 2. Separation of the immunoreactive γ_{11} by reversed-phase HPLC. Fractions containing the immunoreactive γ_{11} were loaded onto a reversed-phase $5C_{18}$-P300 column as described in Section 2. The absorbance at 214 nm of the eluate was monitored continuously. Inset, aliquots of the numbered peak fractions were lyophilized and subjected to Tricine/SDS-PAGE, and the gel was then stained with silver (A) or immunoblotted with antibodies against γ_{11} (B). Numbers on the left indicate molecular masses in kilodaltons.

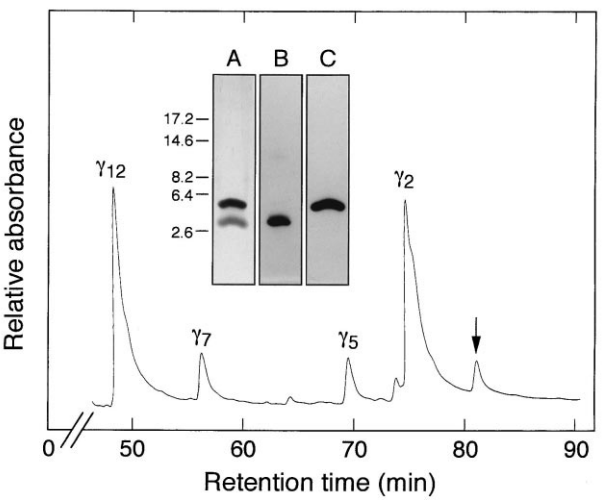

Fig. 3. Separation of the immunoreactive γ_{10} by reversed-phase HPLC. The βγ complexes containing the immunoreactive γ_{10} and other γ subunits were loaded onto a reversed-phase 5PE column as described in Section 2. The absorbance at 214 nm of the eluate was monitored continuously. Inset, aliquots of the peak fraction indicated by an arrow were lyophilized and subjected to Tricine/SDS-PAGE, and the gel was then stained with silver (A) or immunoblotted with antibodies against γ_{10} (B) and γ_2 (C). Numbers on the left indicate molecular masses in kilodaltons. The γ subunits in other peaks were identified by immunoblot with antibodies against various γ and the results were consistent with our previous observations [13,21].

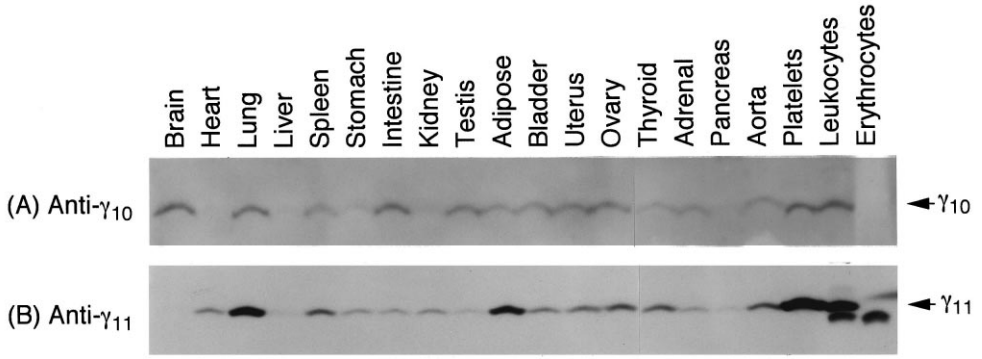

Fig. 4. Tissue distribution of γ_{10} and γ_{11} in the rat. Cholate extracts (A: 50 μg of protein; B: 30 μg of protein) of various rat tissues were subjected to Tricine/SDS-PAGE and immunoblotted with antibodies against γ_{10} and γ_{11}.

from β (Fig. 1), and βγ complexes composed of other γ subunits were bound to the column (data not shown). Then the immunoreactive γ_{11} was subjected to successive reversed-phase HPLC of $5C_8$ and $5C_{18}$-P300 columns under denaturing conditions. As shown in Fig. 2, the immunoreactive γ_{11} was eluted into three peaks, with peak 1 containing large amounts of contaminants. By contrast, peaks 2 and 3 showed a single band, both of which reacted with antibodies against γ_{11} (Fig. 2, inset). Both immunoreactive γ_{11} in peaks 2 and 3 were directly subjected to amino acid sequence analysis, which revealed an identical sequence (PALHIEDLPEKEKLKME-VEQLR) corresponding to the N-terminal sequence of γ_{11} (Pro^2-Arg^{23}) [15], indicating both proteins are the γ_{11}. The two forms of γ_{11} in peaks 2 and 3 may differ in their C-terminal modification (see Section 4).

Fraction II from DEAE-Sephacel containing the immunoreactive γ_{10} was also subjected to successive column chromatography of Ultrogel AcA 34, heptylamine-Sepharose, and TSKgel phenyl-5PW. After the chromatography, immunoreactive γ_{10}-rich fractions still contained large amounts of βγ complexes composed of other γ subunits, mainly because of the low content of γ_{10} in this tissue. For better separation of γ subunits, βγ fractions containing the immunoreactive γ_{10} were subjected to a reversed-phase HPLC of 5PE column under denaturing conditions as described previously [13]. As shown in Fig. 3, the last peak, which followed the large peak of γ_2, contained the immunoreactive γ_{10} in addition to the γ_2. In spite of contamination of γ_2, this fraction was subjected to amino acid sequence analysis. Although the N-terminus of the immunoreactive γ_{10} was blocked, the amino acid sequence of a major fragment (LQQYxMQNAxKDALLVGV-PAGSNPFREPR) produced by *S. aureus* V8 protease coincided exactly with the internal sequence of γ_{10} (Leu^{35}-Arg^{63}) [15]. The γ_{10} showed an apparently low molecular weight on Tricine/SDS-polyacrylamide gels. However, it is unlikely to be due to the degradation of the γ_{10} protein during purification, because the mobility of the purified protein was identical to that of the immunoreactive γ_{10} in crude extracts (data not shown).

Antibodies against γ_{10} and γ_{11} peptides reacted with γ_{10} and γ_{11}, respectively, but not with γ_1, γ_2, γ_3, γ_5, γ_7 or γ_{12} (data not shown). Using these antibodies, the distribution of γ_{10} and γ_{11} in various rat tissues was examined. Both γ subunits were distributed in a wide variety of tissues tested (Fig. 4), consistent with the results obtained with Northern analyses [15]. In contrast to apparently low concentrations of γ_{10}, the γ_{11} was present at relatively high levels in most tissues, but was not detectable in the brain and erythrocytes. It should be noted that γ_{11} was abundant in platelets and leukocytes (Fig. 4). The bands with low molecular weights observed in extracts of leukocytes and erythrocytes with antibodies against γ_{11} are probably non-specific.

4. Discussion

In the present study, we purified two forms of γ subunit from bovine lung and identified them as γ_{10} and γ_{11}. Both γ subunits were detected in most rat tissues, with noticeably high levels of γ_{11} in the platelets. The relatively high concentrations of γ_{10} and γ_{11} were observed in the lung among the various rat tissues. Among the various γ subunits in bovine lung, however, both γ_{10} and γ_{11} are rather minor isoforms when judged with protein staining during purification. Therefore, these two isoforms, especially γ_{10}, are not present at high concentrations in most tissues. In our previous studies, four forms of γ subunit (γ_2, γ_5, γ_7, and γ_{12}) were isolated from fractions containing βγ complexes of bovine lung by reversed-phase HPLC, but γ_{10} and γ_{11} were not detected [13,21]. The reasons why the latter two γ subunits were not isolated in previous studies are as follows: (1) because we previously purified βγ complexes from Fraction I of DEAE-Sephacel chromatography, which did not contain the γ_{10}; and (2) βγ fractions did not contain γ_{11}, because γ_{11} was freed from β.

The C-terminus of γ_{11} is farnesylated like those of retinal γ subunits, γ_1 and γ_c [9,15,17], while the other isoforms are geranylgeranylated [3,13,15,18,19]. Since the biological properties of $\beta\gamma_1$ are appreciably different from those of the other βγ complexes containing geranylgeranylated γ subunits [4–6], $\beta\gamma_{11}$ complex might show unique characteristics. We first intended to purify $\beta\gamma_{11}$ complex, but only free γ_{11} from β subunit was obtained even under non-denaturing conditions. We previously reported that the free form of γ_3 was isolated from bovine brain, but made up only a small portion of the total amount of γ_3 [20]. To examine whether γ_{11} is freed from β in tissue, immunoprecipitation of γ_{11} from the extract of bovine lung with antibodies against γ_{11} was carried out in the buffer containing 1% Triton X-100 and 0.5% sodium cholate. The β subunit was coimmunoprecipitated with γ_{11}, suggesting that at least some γ_{11} associates with β in the tissue (data not shown). However, it is still unclear if the γ_{11} is dissociated from β in the tissues or during purification with 1% sodium

cholate. Taken together, it has been believed that β and γ tightly associated under non-denaturing conditions, but the binding of γ_{11} and γ_3 with β is relatively weak.

With respect to structure, the N-terminal amino acid residue of γ_{11} is an unmodified Pro^2, as it is of γ_1 [17,23], while N-terminal residues of most γ subunits are modified probably by acylation [3,5,21,24]. The γ_{11} was separated into two fractions (peak 2 and peak 3) on reversed-phase HPLC of $5C_{18}$-P300 column. Since the N-termini of both γ_{11} were identical, their C-termini might differ. It is well known that the C-terminal cysteine of γ subunits is isoprenylated and methyl esterified [3,13,15,17–19,25]. Both γ_{11} showed identical mobility on polyacrylamide gel, suggesting that the difference is not due to the lack of an isoprenyl group, which speeds the migration of γ subunits [25]. By contrast, Ohguro et al. [25] showed the separation of methylated and non-methylated forms of γ_1 on the same column used in the present study, and the difference of retention time between two forms of γ_1 was similar to that between two γ_{11}. Therefore, it is most likely that the γ subunits in peak 2 and 3 are non-methylated and methylated forms of γ_{11}, respectively.

Acknowledgements: This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan.

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