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Identification of Lung Major GTP-Binding Protein as G_{i2} and Its Distribution in Various Rat Tissues Determined by Immunoassay†

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ABSTRACT: Antisera were raised in rabbits against the 40-kDa α subunit of bovine lung GTP-binding protein, which were identified as the α subunit of G_{i2} ($G_{i2}\alpha$) by the analysis of the partial amino acid sequence. Antibodies were purified with a $G_{i2}\alpha$-coupled Sepharose column and then were passed through a $G_{i1}\alpha$-coupled Sepharose column to remove antibodies reactive also with 41-kDa α. Purified antibodies reacted with $G_{i2}\alpha$, but not with $G_{i1}\alpha$, $G_{i3}\alpha$, or $G_o\alpha$ in an immunoblot assay. A sensitive enzyme immunoassay method for the quantification of $G_{i2}\alpha$ was developed by using these purified antibodies. The assay system consisted of polystyrene balls with immobilized antibody $F(ab')_2$ fragments and the same antibody Fab' fragments labeled with β-D-galactosidase from *Escherichia coli*. The minimal detection limit of the assay was 1 fmol, or 40 pg. Samples from various tissues were solubilized with 2% sodium cholate and 1 M NaCl, and the concentrations of $G_{i2}\alpha$ were determined. $G_{i2}\alpha$ was detected in all the tissues examined in the rat. The highest concentration was found in platelets and leukocytes when the data were expressed as picomoles per milligram of protein. The spleen, lung, and cerebral cortex contained relatively high levels of $G_{i2}\alpha$. In the bovine brain, $G_{i2}\alpha$ was distributed almost uniformly among the various regions. The concentrations of $G_{i2}\alpha$ were constant in the rat brain throughout ontogenic development, in contrast with those of $G_o\alpha$ which were markedly increased with age.

Among the GTP-binding proteins (G proteins),[1] there are several proteins which can be ADP-ribosylated by pertussis toxin. They include transducin, G_i, and G_o, which are known to be homologous proteins (Gilman, 1987). Transducin is localized in the vertebrate retina and communicates between light activation of rhodopsin and stimulation of cyclic GMP phosphodiesterase. G_o is predominantly localized in nervous tissues and neuroendocrine cells (Sternweis & Robishaw, 1984; Asano et al., 1988c). The functions of G_i and G_o are not fully elucidated yet, but evidence suggests that they are involved not only in inhibition of adenylate cyclase but also in stimulation of phospholipase C and phospholipase A_2 (Gilman, 1987). Some other reports indicate that the K^+ channel or Ca^{2+} channel is regulated by G_i, G_o, or other G proteins. These G proteins are composed of three different subunits, α, β, and γ. The α subunit is unique to each G protein, while the β and γ subunits are considered to be identical with or very similar

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[1] Abbreviations: G protein, GTP-binding protein; G_i, inhibitory GTP-binding protein of adenylate cyclase; G_o, related GTP-binding protein of unknown function; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TPCK, tosylphenylalanyl chloromethyl ketone; kDa, kilodalton(s); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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to each other (Gilman, 1987). The molecular weights of α subunits of G_i and G_o were reported to be 41 000 and 39 000, respectively. However, other pertussis toxin substrates, whose α subunits have a molecular weight of 40 000, have recently been found in various tissues (Katada et al., 1987; Gierschik et al., 1987; Oinuma et al., 1987; Dickey et al., 1987; Morishita et al., 1988). On the other hand, the studies of molecular cloning and sequence analysis of cDNAs revealed the presence of multiple forms of the α subunit of G_i (Nukada et al., 1986; Itoh et al., 1986, 1988b; Sullivan et al., 1986; Michel et al., 1986; Didsbury et al., 1987; Van Meurs et al., 1987; Didsbury & Snyderman, 1987; Bray et al., 1987; Suki et al., 1987; Jones & Reed, 1987; Beals et al., 1987). They are designated as $G_{i1}\alpha$, $G_{i2}\alpha$, and $G_{i3}\alpha$ (Gilman, 1987; Suki et al., 1987). The analysis of the partial amino acid sequence revealed that brain 41-kDa α and 40-kDa α were identical with $G_{i1}\alpha$ and $G_{i2}\alpha$, respectively (Itoh et al., 1988a). More recently, 41-kDa α from HL60 cells (Goldsmith et al., 1988) and 40-kDa α from human erythrocytes (Codina et al., 1988) were identified as $G_{i3}\alpha$.

In our previous paper, we have purified two α subunits of pertussis toxin sensitive G proteins from bovine lung and raised antisera in rabbits against a major α subunit with a molecular weight of 40K (Morishita et al., 1988). This protein has been identified as $G_{i2}\alpha$ by the analysis of the partial amino acid sequence in this paper. To elucidate the physiological significance of G_{i2}, we have established a sensitive enzyme immunoassay method with the purified antibodies for quantifying $G_{i2}\alpha$ and investigated the localization of $G_{i2}\alpha$ in various rat tissues.

Experimental Procedures

Purification of G Proteins and Antibodies. Bovine lung G proteins and rat 40-kDa α subunit were purified according to the method described previously (Morishita et al., 1988). $G_{i1}\alpha$ (termed as $G_i\alpha$ previously) and $G_o\alpha$ were purified from bovine or rat brain by the method described in Asano et al. (1988a).

The antisera were raised in rabbits by intracutaneous injection of the purified bovine 40-kDa α (0.15 mg per rabbit) in Freund's complete adjuvant, and the antibodies were purified from the antisera by use of the antigen coupled to a Sepharose column as described in Morishita et al. (1988). The antibodies were then passed through a $G_{i1}\alpha$–Sepharose column to adsorb antibodies which reacted with 41-kDa α (Morishita et al., 1988).

Analysis of Partial Amino Acid Sequence. A sample containing approximately 0.5 nmol of purified bovine lung 40-kDa α or 41-kDa α, 20 mM Hepes (pH 8.0), 0.1 mM EDTA, 0.5 mM dithiothreitol, 0.1% Lubrol PX, and 0.1 M potassium phosphate was heated at 65 °C for 15 min and digested at 37 °C with tosylphenylalanyl chloromethyl ketone (TPCK)-treated trypsin. TPCK-treated trypsin was added at an α-subunit/trypsin ratio of 40 (w/w). After incubation for 12 h, the same amount of TPCK-treated trypsin was added, and the incubation was continued for an additional 12 h. Purification of tryptic peptides was carried out by the method described previously (Itoh et al., 1988a) with reverse-phase high-performance liquid chromatography (HPLC). Several fractions were subjected to amino acid sequence analysis with a gas-phase sequencer (Model 477A, Applied Biosystems). Phenylthiohydantoin-amino acids were analyzed by HPLC (Model 120A, Applied Biosystems).

Immunoassay System and Assay Procedures. In order to increase the sensitivity of the assay (Kato et al., 1976) and to protect the assay from sample interference (Kato et al., 1979), we prepared the immunoassay system with antibody $F(ab')_2$ and Fab' fragments. The purified IgG antibody was digested with pepsin from porcine stomach mucosa (Sigma Chemical Co.), and the resulting $F(ab')_2$ fragments were isolated by passing the mixture through a Sephadex G-150 column (Kato et al., 1976). The sandwich-type enzyme immunoassay system was composed of polystyrene balls (3.18 mm in diameter; Precision Plastic Ball Co., Chicago, IL) with immobilized antibody $F(ab')_2$ fragments, and the same antibody Fab' fragments were labeled with β-D-galactosidase from *Escherichia coli*. Details of immobilization of antibodies on polystyrene balls (Kato et al., 1977) and labeling of antibodies with β-D-galactosidase (Kato et al., 1976) are described elsewhere.

Procedures of the immunoassay were almost the same as those described for the assay of $G_o\alpha$ (Asano et al., 1987). A polystyrene ball with antibodies was incubated with shaking at 30 °C for 5 h with samples or standard 40-kDa α in a final volume of 0.5 mL of buffer G [10 mM sodium phosphate buffer, pH 7.0, 0.3 M NaCl, 1 mM $MgCl_2$, 0.1% bovine serum albumin, 0.5% protease-treated gelatin (Kato et al., 1980), and 0.1% NaN_3] in a test tube (10 × 75 mm). Each ball was washed twice with 1 mL of buffer A (10 mM sodium phosphate, pH 7.0, 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% bovine serum albumin, and 0.1% NaN_3), transferred into a fresh test tube with 0.2 mL of buffer A containing 2 milliunits (expressed as units of galactosidase activity; 1 unit = 1 μmol of product/min under the assay conditions) of galactosidase-labeled antibodies, and left standing at 4 °C overnight. After unbound label was washed off, the galactosidase activity on the ball was assayed with a fluorogenic substrate, 4-methylumbelliferyl β-D-galactoside (Sigma Chemical Co.), as described by Kato et al. (1977). For the immunoassays with extracts from bovine or rat tissues, the 40-kDa α purified from the tissue of the respective animal was used as a standard.

Preparation of Tissue Extracts. Adult bovine brains were obtained at a local slaughterhouse within 30 min of death and chilled in ice. Portions of about 1 g each of various regions of the tissue were dissected. For all the experiments with the rats, Wistar rats were used. The tissues were obtained from 3-month-old male rats for the determination of the distribution of 40-kDa α. Only the uterus and ovary were sampled from female rats. The erythrocytes, leukocytes, and platelets were separated from rat blood obtained by venipuncture with heparin (15 units/mL) according to the method of Skoog and Beck (1956). The mononuclear leukocytes were separated from the blood by the method of Boyum (1968) using Ficoll–Paque (Pharmacia LKB Biotechnology). All tissue samples were stored at −80 °C until analysis. The frozen tissue was homogenized at 0 °C with a Potter–Elvehjem homogenizer in 4.5 volumes (v/w) of 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 5 mM β-mercaptoethanol. Then 10% sodium cholate and 4 M NaCl were added to final concentrations of 2% and 1 M, respectively, and the tissue was further homogenized. The homogenate (~2 mL) was sonicated for 1 min and then centrifuged at 4 °C at 100000g for 1 h, and the supernatant fraction was used for the analysis after dilution with buffer G (Asano et al., 1987).

Proteins were determined by the method of Schaffner and Weissmann (1973) with bovine serum albumin as the standard.

Results

Identification of Bovine Lung 40-kDa α as $G_{i2}\alpha$. Purified bovine lung 40-kDa α was digested with TPCK–trypsin, and digested peptides were separated by reverse-phase HPLC. The elution profile of tryptic peptides on HPLC was similar to that observed with brain 40-kDa α (Itoh et al., 1988a). Four fractions (I–IV) were subjected to amino acid sequence

bovine	**40 kDa α (I-1)**		**K/R Y D E A A S Y I Q S K**
human	Gi2α	(296-307)	K Y D E A A S Y I Q S K
rat	Gi2α	(296-307)	K Y D E A A S Y I Q S K
mouse	Gi2α	(296-307)	K Y D E A A S Y I Q S K
porcine	G40α	(I-1)	K/R Y D E A A S Y I Q
human	Gi3α	(295-306)	T Y E E A A A Y I Q C Q
rat	Gi3α	(295-306)	T Y E E A A A Y I Q C Q
human	Gi1α	(295-306)	T Y E E A A A Y I Q C Q
bovine	Gi1α	(295-306)	T Y E E A A A Y I Q C Q
rat	Gi1α	(295-306)	T Y E E A A A Y I Q C Q
rat	Goα	(296-307)	T Y E D A A A Y I Q T Q
bovine	Goα	(296-307)	T Y E D A A A Y I Q A Q

bovine	**40 kDa α (II)**		**I A Q S D Y I P T Q Q D V L R**
human	Gi2α	(163-177)	I A Q S D Y I P T Q Q D V L R
rat	Gi2α	(163-177)	I A Q S D Y I P T Q Q D V L R
mouse	Gi2α	(163-177)	I A Q S D Y I P T Q Q D V L R
porcine	G40α	(II)	I A Q S D Y I P T Q Q D V L R
human	Gi3α	(162-176)	I S Q S N Y I P T Q Q D V L R
rat	Gi3α	(162-176)	I S Q T N Y I P T Q Q D V L R
human	Gi1α	(162-176)	I A Q P N Y I P T Q Q D V L R
bovine	Gi1α	(162-176)	I A Q P N Y I P T Q Q D V L R
rat	Gi1α	(162-176)	I A Q P N Y I P T Q Q D V L R
rat	G41α	(I)	I A Q P N Y I P T Q Q X V L
rat	Goα	(163-177)	I G A A D Y Q P T E Q D I L R
bovine	Goα	(163-177)	I G A A D Y Q P T E Q D I L R

bovine	**40 kDa α (IV)**		**E Y Q L N D S A A Y Y L N D L E R**
human	Gi2α	(146-162)	E Y Q L N D S A A Y Y L N D L E R
rat	Gi2α	(146-162)	E Y Q L N D S A A Y Y L N D L E R
mouse	Gi2α	(146-162)	E Y Q L N D S A A Y Y L N D L E R
porcine	G40α	(IV)	E Y Q L N D S A A Y Y L N D L E R
human	Gi3α	(145-161)	E Y Q L N D S A S Y Y L N D L D R
rat	Gi3α	(145-161)	E Y Q L N D S A S Y Y L N D L D R
human	Gi1α	(145-161)	E Y Q L N D S A A Y Y L N D L D R
bovine	Gi1α	(145-161)	E Y Q L N D S A A Y Y L N D L D R
rat	Gi1α	(145-161)	E Y Q L N D S A A Y Y L N D L D R
rat	G41α	(II)	E Y Q L N D S A A Y Y L N D L X R
bovine	Goα	(146-162)	E Y Q L N D S A K Y Y L D S L D R
rat	Goα	(146-162)	E Y Q L N D S A K Y Y L D S L D R
rat	G39α	(I & II)	E Y Q L N D S A Y Y L D S L D

FIGURE 1: Amino acid sequence homology among the G-protein α subunits. The sequences of the G-protein α subunits have been taken from the literature: human $G_{i2}\alpha$ (Itoh et al., 1988b); rat $G_{i2}\alpha$ (Itoh et al., 1986); mouse $G_{i2}\alpha$ (Sullivan et al., 1986); human and rat $G_{i3}\alpha$'s (Itoh et al., 1988b); human $G_{i1}\alpha$ (Bray et al., 1987; Itoh et al., 1988b); bovine $G_{i1}\alpha$ (Nukada et al., 1986); rat $G_{i1}\alpha$ (Jones & Reed, 1987); rat $G_o\alpha$ (Itoh et al., 1986); bovine $G_o\alpha$ (Van Meurs et al., 1987). The partial amino acid sequences of rat 41-kDa (G41α), 39-kDa (G39α), and porcine 40-kDa (G40α) proteins are described in Itoh et al. (1986, 1988a). In the predicted amino acid sequences, amino acid residues are numbered beginning with the initiating methionine of the respective polypeptides. The two sequences (G39α-I and G39α-II) of rat 39-kDa protein are EYQLNDSA and YYLDSLD, respectively. In the bovine 40-kDa α (I-1) and the porcine G40α (I-1) sequences, the NH_2-terminal amino acid residues are assumed to be K or R (K/R). X represents unidentified residues. Differences, as compared with the residues of the bovine lung 40-kDa α, are boxed.

analysis. Fractions II and IV contained a single peptide, while fraction I was a mixture of three peptides and fraction III was a mixture of two peptides. The sequences determined were as follows: I-1, YDEAASYIQSK; I-2, MFDVGGQR; I-3, DLFEEK; II, IAQSDYIPTQQDVLR; III-1, TTGIVETHFTFK; III-2, LLLLGAGESGK; IV, EYQLNDSAAYYLNDLER. Assignment of the sequences of the three or two peptides in fraction I or III, respectively, was based on the predicted amino acid sequences from the known G-protein α-subunit genes and cDNAs (Nukada et al., 1986; Itoh et al., 1986, 1988b; Sullivan et al., 1986; Michel et al., 1986; Didsbury et al., 1987; Van Meurs et al., 1987; Didsbury & Snyderman, 1987; Bray et al., 1987; Suki et al., 1987; Jones & Reed, 1987; Beals et al., 1987). We compared the partial amino acid sequences derived from bovine lung 40-kDa α with those predicted from G-protein α-subunit genes and cDNAs (Figure 1). The sequences of I-2, I-3, III-1, and III-2 matched completely those which were identical in all different members of the $G_i\alpha$ subfamily (not shown) (Itoh et al., 1988b). In contrast, the sequences of I-1, II, and IV were identical with those of human (Itoh et al., 1988b), rat (Itoh et al., 1986), and mouse (Sullivan et al., 1986) $G_{i2}\alpha$, but not with those of $G_{i1}\alpha$, $G_{i3}\alpha$, and $G_o\alpha$. The partial amino acid sequences derived from bovine lung 40-kDa α were also identical with those from porcine brain 40-kDa α (Itoh et al., 1988a). Therefore, bovine lung 40-kDa α can be expressed as $G_{i2}\alpha$.

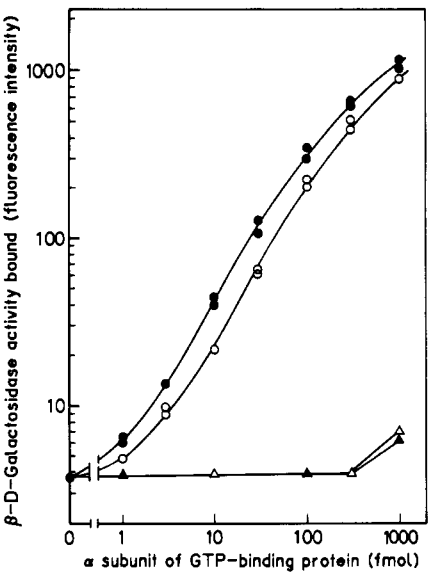

FIGURE 2: Standard curves of the enzyme immunoassay for bovine and rat $G_{i2}\alpha$ and cross-reactivity of the assay with $G_{i1}\alpha$ and $G_{i3}\alpha$. Various amounts of bovine $G_{i2}\alpha$ (●), rat $G_{i2}\alpha$ (○), rat $G_{i1}\alpha$ (△), and bovine $G_{i1}\alpha/G_{i3}\alpha$ mixture (▲) were subjected in duplicate to the immunoassay. β-D-Galactosidase activity bound on the polystyrene ball is expressed as the fluorescence intensity of 4-methylumbelliferone produced in a 20-min reaction with 0.1 mM 4-methylumbelliferyl β-D-galactoside. In fluorescence intensity, 1000 was defined as that by 1 μM 4-methylumbelliferone.

In addition to 40-kDa α, we also analyzed the partial amino acid sequence of 41-kDa α which were purified as a minor G protein from bovine lung (Morishita et al., 1988). The analysis of the 8 tryptic peptides containing 93 amino acid residues revealed that bovine lung 41-kDa α was a mixture of $G_{i1}\alpha$ and $G_{i3}\alpha$ and the ratio of $G_{i1}\alpha$ to $G_{i3}\alpha$ was 2–3 (not shown).

Specificity of Antibodies. The reactivity of the antibodies against 40-kDa α with purified bovine G proteins was described in Morishita et al. (1988); the antibodies reacted only with lung 40-kDa α ($G_{i2}\alpha$) but not with brain 41-kDa α ($G_{i1}\alpha$), lung 41-kDa α ($G_{i1}\alpha$ and $G_{i3}\alpha$), or brain 39-kDa α ($G_o\alpha$). The antibodies also reacted with rat $G_{i2}\alpha$ but did not cross-react with rat $G_{i1}\alpha$ or $G_o\alpha$ in an immunoblot assay (not shown).

Detection Limit, Specificity, and Precision of the Immunoassay for Bovine and Rat $G_{i2}\alpha$. Standard curves for the assay of bovine and rat $G_{i2}\alpha$ and cross-reactivity of the assay with rat $G_{i1}\alpha$ or bovine $G_{i1}\alpha/G_{i3}\alpha$ mixture were shown in Figure 2. The detection limit, defined as the lowest concentration giving galactosidase activity significantly different from that of the zero standard at the 0.99 confidence level, was 1 fmol (40 pg) for the bovine $G_{i2}\alpha$ assay. The cross-reactivity of rat $G_{i2}\alpha$ in the immunoassay was about 60% of bovine $G_{i2}\alpha$. The assay did not cross-react with $G_{i1}\alpha$, $G_{i3}\alpha$ (Figure 2), $G_o\alpha$, or $\beta\gamma$ (not shown). These results indicate that the present immunoassay method is highly sensitive for the specific quantification of $G_{i2}\alpha$.

Samples from various rat tissues were solubilized with 2% sodium cholate and 1 M NaCl (Asano et al., 1987), and concentrations of $G_{i2}\alpha$ were determined by the immunoassay. The efficiency of the cholate extraction procedure was checked by immunoblots of cholate-extracted membranes solubilized with SDS. No band was observed on immunoblots with several tissues tested, a finding suggesting that most of the $G_{i2}\alpha$ in the membranes was extracted by cholate (not shown).

The precision of the assay was tested by assaying 3 samples of bovine and rat tissue extracts 15 times in 1 assay (within-assay) or in duplicate in 12 consecutive assays (between-assay).

Table I: Distribution of $G_{i2}\alpha$ and $G_o\alpha$ in Various Rat Tissues[a]

tissue	$G_{i2}\alpha$ pmol/mg of protein	$G_{i2}\alpha$ pmol/g of tissue	$G_o\alpha$[b] (pmol/g of tissue)
spleen	7.27 ± 2.78	889 ± 291	8 ± 6
lung	6.66 ± 2.21	559 ± 176	39 ± 9
cerebral cortex	6.38 ± 1.69	448 ± 80	7710 ± 1520
uterus	5.36 ± 1.91	384 ± 90	8 ± 1
ovary	4.99 ± 1.51	316 ± 64	14 ± 5
cerebellum	4.60 ± 0.85	331 ± 47	4440 ± 1490
testis	3.95 ± 1.19	250 ± 56	26 ± 10
small intestine	3.74 ± 0.56	157 ± 37	29 ± 13
pituitary gland	3.63 ± 0.71	432 ± 106	517 ± 54
adipose tissue	3.07 ± 0.95	28 ± 6	5 ± 2
large intestine	2.88 ± 0.58	226 ± 37	55 ● 13
urinary bladder	2.82 ± 0.94	156 ± 59	84 ± 19
adrenal gland	2.66 ± 0.61	277 ± 54	21 ± 9
stomach	2.57 ± 0.65	211 ± 50	97 ± 11
kidney	2.44 ± 0.53	264 ± 61	21 ± 9
liver	1.05 ± 0.20	156 ± 34	3 ± 1
heart	0.84 ± 0.20	119 ± 26	62 ± 16
pancreas	0.75 ± 0.14	81 ± 25	12 ± 6
leg muscle	0.29 ± 0.17	33 ± 20	5 ± 2
platelets	13.6 ± 1.6	8.31 ± 3.27[c]	ND[e]
leukocytes	8.53 ± 0.57	235 ± 65[c]	ND
erythrocytes	0.11 ± 0.02	1.49 ± 0.27[c]	ND
plasma	0.001 ± 0.001	0.06 ± 0.03[d]	ND

[a] The cholate extract from each tissue was subjected to the immunoassay of $G_{i2}\alpha$ and protein assay. Data are mean ± SD values from five rats. [b] Data are from Asano et al. (1988c). [c] Picomoles per 10^9 cells. [d] Picomoles per milliliter. [e] ND, no detected.

The coefficients of variation in each assay were <15%. The analytical recovery of $G_{i2}\alpha$ (100 fmol) added to the immunoassay together with the extract was ~100% (103 ± 9%, n = 8), a result indicating that there are no inhibitory substances in the extract under the assay conditions.

For determination of the applicability of the immunoassay for measuring $G_{i2}\alpha$ content in the crude extract, several samples of the bovine and rat tissue extract were serially diluted, and aliquots of samples containing various volumes of original extracts were subjected to the immunoassay. The amounts of $G_{i2}\alpha$ measured in each extract were proportional to the volumes of the extract used for the assay, an observation indicating that the concentrations of $G_{i2}\alpha$ can be determined by this assay.

Quantification of $G_{i2}\alpha$ in Various Rat Tissues. Table I shows the concentrations of $G_{i2}\alpha$ in rat various tissues, exprssed as picomoles per gram of tissue and picomoles per milligram of solubilized protein. The concentrations of $G_o\alpha$ in the same tissues determined by using the enzyme immunoassay method were those reported previously (Asano et al., 1988c). $G_{i2}\alpha$ was detected in all the tissues examined, and in contrast to $G_o\alpha$, it was widely distributed with relatively high concentrations. The highest concentration was found in platelets and leukocytes in the blood when the data were expressed as picomoles per milligram of protein. Because the lymphocytes are major populations in rat leukocytes, the mononuclear cells isolated and their cholate extracts were subjected to the immunoassay. The concentration of $G_{i2}\alpha$ (218 ± 26 pmol/10^9 cells or 7.76 ± 0.47 pmol/mg of protein) was a little less than that in the total leukocyte fraction, suggesting that polymorphonuclear cells contained a little more $G_{i2}\alpha$ than mononuclear cells. The spleen, lung, and cerebral cortex contained relatively high concentrations of $G_{i2}\alpha$. The $G_{i2}\alpha$ could be detected even in the blood plasma. An immunoblot of the membranes prepared from several tissues probed with purified anti-$G_{i2}\alpha$ antibody showed that all the membranes examined had the immunoreactivity at M_r 40000 corresponding to $G_{i2}\alpha$ (not shown).

Table II: Regional Distribution of $G_{i2}\alpha$ in Bovine Brain[a]

region	$G_{i2}\alpha$ pmol/g of tissue	$G_{i2}\alpha$ pmol/mg of protein
cerebral cortex	644 ± 153	14.0 ± 2.9
cerebral white matter	428 ± 79	11.1 ± 2.1
hippocampus	488 ± 40	13.2 ± 1.9
thalamus	500 ± 99	11.2 ± 1.0
nucleus caudatus	647 ± 149	11.4 ± 2.4
superior colliculus	368 ± 104	8.4 ± 1.7
inferior colliculus	395 ± 120	10.6 ± 2.6
cerebellar cortex	551 ± 195	13.6 ± 3.9
cerebellar white matter	300 ± 74	8.1 ± 1.7
medulla oblongata	337 ± 103	10.2 ± 3.3
optic tract	293 ± 47	9.0 ± 1.0
spinal cord	280 ± 51	9.9 ± 1.5

[a] The cholate extract from each sample was subjected to the immunoassay of $G_{i2}\alpha$ and protein assay. Data are mean ± SD values from five animals.

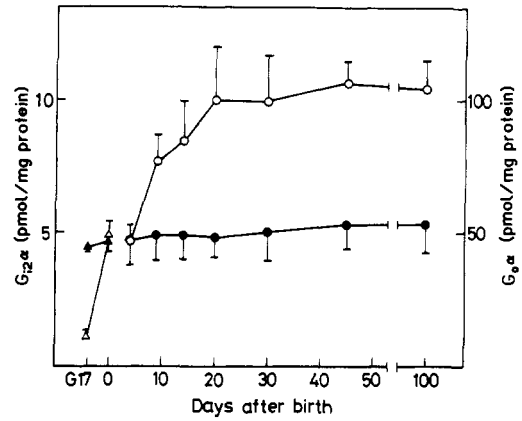

FIGURE 3: Ontogeny of $G_{i2}\alpha$ and $G_o\alpha$ in rat brain. The concentrations in whole brains were measured for rats at 17 days of gestation (G17) and the day of birth (▲, △), and those in the cerebral cortex (●, ○) were measured for rats older than 4 days after birth. The concentrations of $G_{i2}\alpha$ (▲, ●), or $G_o\alpha$ (△, ○) are expressed as picomoles per milligram of protein. Data are mean ± SD values from five rats.

An immunohistochemical study showed that $G_{i2}\alpha$ was largely distributed on the cell membranes of many types of cells, including neuropil in the brain, type I alveolar epithelial cells and blood capillaries in the lung, and lymphocytes, sinus epithelial cells, and reticular cells in the spleen (not shown). In whole blood, $G_{i2}\alpha$ immunoreactivity was associated with polymorphonuclear and mononuclear leukocytes and platelets, but not with erythrocytes (not shown).

Quantification of $G_{i2}\alpha$ in Bovine Brain. Table II shows the concentrations of $G_{i2}\alpha$ in the cholate extracts from various regions of bovine brain, expressed in picomoles per gram of tissue and picomoles per milligram of protein. The $G_{i2}\alpha$ was distributed more uniformly than the $G_o\alpha$ was (Asano et al., 1987).

Ontogeny of $G_{i2}\alpha$ in Rat Brain. We have previously reported the ontogeny of $G_o\alpha$ in rat brain (Asano et al., 1988b). The level of $G_o\alpha$ increased with age from embryo and reached the adult level at approximately 20 days after birth (Asano et al., 1988b) (Figure 3). When the ontogeny of $G_{i2}\alpha$ was determined in rat brain, the level of $G_{i2}\alpha$ was almost constant at any age examined in contrast to that of $G_o\alpha$ (Figure 3).

Discussion

We have previously purified two pertussis toxin sensitive G proteins from bovine lung and raised antisera against the 40-kDa α subunit of the major G protein (Morishita et al., 1988). In this study, we developed an enzyme immunoassay

method by the use of the purified antibodies to 40-kDa α. Because the presence of the $G_i\alpha$ subfamily has been revealed by studies of molecular cloning of G-protein α-subunit gene and cDNAs, we first identified bovine lung 40-kDa α by the analysis of the partial amino acid sequences. We determined the partial amino acid sequences of the 7 tryptic peptides containing 80 amino acid residues of bovine 40-kDa α. The results indicated that the bovine 40-kDa α was different from $G_{i1}\alpha$, $G_{i3}\alpha$, and $G_o\alpha$, but identical with $G_{i2}\alpha$ (Figure 1). The 40-kDa α was also purified from rat brain and used as the standard in the immunoassay of the extracts from rat tissues. This protein seemed to be $G_{i2}\alpha$, because it cross-reacted with anti-$G_{i2}\alpha$ antibodies, porcine brain 40-kDa α was already identified to be $G_{i2}\alpha$ (Itoh et al., 1988a), and strong conservation of the amino acid sequence in each group of the G-protein α-subunit family was found (Nukada et al., 1986; Itoh et al., 1986, 1988b; Sullivan et al., 1986; Michel et al., 1986; Didsbury et al., 1987; Van Meurs et al., 1987; Didsbury & Snyderman, 1987; Bray et al., 1987; Suki et al., 1987; Jones & Reed, 1987; Beals et al., 1987).

The immunoassay method prepared with these antibodies is sensitive and specific to $G_{i2}\alpha$. The minimal detection limit of the assay is 1 fmol, or 40 pg, of $G_{i2}\alpha$ under the present assay conditions. The assay scarcely cross-reacts with $G_{i1}\alpha$, $G_{i3}\alpha$, or $G_o\alpha$. Because the antibodies cross-react with $G_{i2}\alpha$ from other species, including rat and human (Morishita et al., 1988), this immunoassay method is available for quantification of $G_{i2}\alpha$ in other species. The antibodies reacted only with the 40-kDa protein in cholate extracts from various rat tissues. However, the cross-reactivity of the antibodies with the newly cloned G_x (Matsuoka et al., 1988) has not been excluded.

$G_{i2}\alpha$ was extracted from the membranes of various tissues by the same procedure as the extraction of $G_o\alpha$ (2% sodium cholate plus 1 M NaCl). When cholate-extracted membranes were solubilized with SDS and subjected to SDS–PAGE followed by immunoblotting in a check of the efficiency of the cholate extraction procedure, no band was observed on immunoblots with several tissues tested. These results suggest that most of the $G_{i2}\alpha$ in the membranes of tissues was extracted by cholate plus NaCl.

$G_{i2}\alpha$ was detected in all the tissues examined, and in contrast to $G_o\alpha$, it was widely distributed with relatively high concentrations. Highest concentrations of $G_{i2}\alpha$ were observed in platelets and leukocytes. The spleen, which produces lymphocytes, also contained a high concentration of $G_{i2}\alpha$. However, similar levels of $G_{i2}\alpha$ were observed in the lung and cerebral cortex, and the immunohistochemical study showed $G_{i2}\alpha$ was associated with the neuropil in the brain and the cell membranes of the type I alveolar epithelial cells and blood capillaries in the lung. In the bovine brain, $G_{i2}\alpha$ was distributed much more uniformly than $G_o\alpha$. Brann et al. (1987) and Kim et al. (1988) reported that $G_{i2}\alpha$ mRNA was expressed in all tissues examined, and Itoh et al. (1988b) suggested that the $G_{i2}\alpha$ gene seemed to be a housekeeping gene. These reports might be confirmed by the uniform distribution of $G_{i2}\alpha$ shown in the present study.

The ontogeny of $G_{i2}\alpha$ in rat brain showed a sharp contrast to that of $G_o\alpha$. The concentrations of $G_{i2}\alpha$ were almost constant at any age examined, whereas those of $G_o\alpha$ were markedly increased with age and the increase seemed to be parallel with synapse formation in the central nervous system (Asano et al., 1988b). These results suggest that G_{i2} is involved in the fundamental process common to the various cellular function.

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Disulfide Bonds Are Localized within the Short Consensus Repeat Units of Complement Regulatory Proteins: C4b-Binding Protein†

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ABSTRACT: Several plasma and membrane proteins belong to a superfamily of structurally related proteins that contain internal homology of a variable number (2–30) of repeating units. Each SCR (short consensus repeat) unit is approximately 60 amino acid residues in length, with the positions of 1 Trp, 2 Pro, and 4 Cys residues being conserved. The aim of this study was to provide experimental evidence that each SCR may exist as an independent structural domain maintained by disulfide bonds. The well-characterized C4b-binding protein (C4BP) with eight SCR units in each of its seven identical chains was chosen for this study. Analysis of the disulfide-bonding pattern indicated that intrachain disulfide bonds may be localized within each SCR unit, with the first and third and the second and fourth half-cystines in each unit being linked. This pattern of disulfides may confer to C4BP (and to other structurally related proteins) a conformation which apparently allows the assembly of the SCR units (4–30) in a tandem fashion. Such an arrangement of the polypeptide chain(s) may explain, in part, the elongated shape of these protein molecules. The structural motif of the SCR units of C4BP is discussed in relation to those previously described for the type II domain of fibronectin and the kringle structure present in various proteins of the coagulation system.

Activation of C3, the central component of the complement system, is under the regulation of at least six proteins. These include plasma proteins factor H (Whaley & Ruddy, 1976) and C4b-binding protein (Fujita et al., 1978), and four membrane-bound proteins: receptor CR1[1] (Fearon, 1980; Medof et al., 1982), receptor CR2 (Moore et al., 1987; Weis et al., 1988), decay accelerating factor (Nicholson-Weller et al., 1982; Kinoshita et al., 1986), and membrane cofactor protein (Lublin et al., 1988). All six proteins are genetically linked in man, being under control of the RCA (regulator of complement activation) locus on human chromosome 1 (Rodriguez de Cordoba et al., 1985, 1988; Rey-Campos et al., 1988; Carroll et al., 1988; Lublin et al., 1988). They also share many features with respect to their biochemical modes of action (Holers et al., 1985).

Each of these proteins is largely composed of a varying number of short consensus repeat (SCR) units, approximately 60 amino acid residues long, in which the positions of 1 tryptophan, 2 prolines, 4 half-cystines, and some other residues are conserved (Reid et al., 1986). The SCR is found also in several other proteins (Table I), although it does not always constitute a large fraction of the total protein sequence. Data on factor B (Campbell et al., 1984), IL-2 receptor (Leonard et al., 1985), C4b-binding protein (Lintin et al., 1987), and factor H (Vik et al., 1988) suggest that each SCR unit is

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[1] Abbreviations: SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; MSH, β-mercaptoethanol; TFA, trifluoroacetic acid; HPLC, high-pressure liquid chromatography; FPLC, fast protein liquid chromatography; Gdn·HCl, guanidine hydrochloride; C4BP, C4b-binding protein, a cofactor for cleavage of the C4bα-chain; factor H and/or CR1, cofactors for factor I mediated cleavages of the C3bα-chain; CR1, a C3b receptor on erythrocytes and other blood cells; CR2, a C3d receptor on B-lymphocytes; DAF, decay accelerating factor present on membranes of blood and tissue cells; MCP, membrane cofactor protein; B and C2, proenzymes in the complement system; C1r and C1s, subcomponents of the first component of complement; β_2I, β_2-glycoprotein I; IL-2(R), receptor for interleukin 2; factor XIII, plasma transglutaminase; SCR, short consensus repeats in C4BP and other related proteins.

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