Biochem. J. (1991) 274, 439–444 (Printed in Great Britain)

Ontogeny of guanine-nucleotide-binding regulatory proteins in rabbit liver

Yumiko KAWAI and Ifeanyi J. ARINZE*
Department of Biochemistry, Meharry Medical College, Nashville, TN 37208, U.S.A.

Ontogeny of trimeric GTP-binding regulatory proteins (G-proteins) and their subunits in rabbit liver during neonatal development was studied, by using bacterial-toxin-catalysed ADP-ribosylation of membrane proteins, immunoblot analysis to quantify the α-subunit (α_s and α_i) of stimulatory (G_s) and inhibitory (G_i) G-protein and the β-subunit, and reconstitution assay with cyc$^-$ membranes (from G_s-deficient variant of S49 lymphoma cell) to measure G_s activity. Under optimal conditions of ADP-ribosylation, little cholera-toxin substrate (α_s) was detected in membranes from liver of neonatal animals up to 24 h of age. Thereafter ribosylatable α_s proteins, i.e. 45 kDa ($\alpha_{s\text{-}1}$) and 52 kDa ($\alpha_{s\text{-}2}$) proteins, were increasingly evident, reaching maximal levels in membranes from animals aged 4–6 weeks. The concentrations of $\alpha_{s\text{-}1}$ and $\alpha_{s\text{-}2}$, as determined by immunoblotting, were 6.1 ± 0.8 and 2.7 ± 0.4 pmol/mg of protein respectively at birth, and did not change during 0–24 h after birth. Thereafter they gradually increased to maximal levels of 22.1 ± 1.3 and 10.5 ± 0.7 pmol/mg of protein for $\alpha_{s\text{-}1}$ and $\alpha_{s\text{-}2}$ respectively, within 6 weeks. The β-subunit also showed a similar 3–4-fold increase during the same age span. In contrast, the pertussis-toxin substrate (α_i) was clearly evident even in membranes from term animals and in all age groups studied. Its developmental pattern, as assessed by ADP-ribosylation, was the same as that determined by immunoblot analysis. The functional activity of G_s in cholate extracts of membranes exhibited similar developmental pattern to that of cholera-toxin-mediated labelling. This activity also paralleled the concentrations of α_s as measured by immunoblotting. These results suggest differential expression of G-protein subunits in liver during neonatal development.

INTRODUCTION

Hormonal activation or inhibition of adenylate cyclase is mediated by guanine nucleoside triphosphates via interaction with distinct stimulatory (G_s) and inhibitory (G_i) guanine-nucleotide-regulatory (G-) proteins (Gilman, 1987; Birnbaumer *et al.*, 1987; Neer & Clapham, 1988; Freissmuth *et al.*, 1989). Both G_s and G_i are heterotrimers which have different α-subunits (α_s and α_i) and almost similar β- (35 and 36 kDa) and γ- (< 10 kDa) subunits (Gilman, 1987; Amatruda *et al.*, 1988). Subspecies heterogeneity also exists within each G-protein subunit class (Jones & Reed, 1987; Itoh *et al.*, 1988; Lang, 1989; Robishaw *et al.*, 1989; Strathmann *et al.*, 1989). Because of the emerging idea that specific, albeit as yet undefined, regulatory interactions may occur between G_s and G_i complexes (Gilman, 1987; Skorecki *et al.*, 1987; Watkins *et al.*, 1989), it may be speculated that the amounts and/or the relative ratios of G-protein subunits may exert control on specific metabolic processes in tissues.

In our studies on the ontogeny of the hepatic adenylate cyclase system, it appeared that uncoupling of the catalytic unit from the β-adrenergic receptor in liver membranes isolated from neonatal rabbits may be related to alterations in amounts of G_s (Kawai *et al.*, 1985). In an attempt to confirm whether quantitative changes occur in the guanine-nucleotide regulatory components during development, we have measured the content of G_s as well as G_i in rabbit liver membranes, using cholera-toxin- and pertussis-toxin-catalysed ADP-ribosylation of membrane proteins. Specific antibodies against α_s, α_i and the β-subunit of the G proteins were also used to determine the concentrations of the subunits of G_s and G_i. The functional activity of G_s in the membranes was measured by reconstitution of detergent extracts of the membranes with cyc$^-$ membranes (from G_s-deficient variant of S49 lymphoma cell).

EXPERIMENTAL

Animals and materials

New Zealand White male (1.2–2.0 kg body wt.) and pregnant female (24–28 days of gestation) rabbits were purchased from Myrtle's Rabbitry, Thompson Station, TN, U.S.A., and fed *ad libitum*. Term fetuses were allowed to be delivered naturally, and the newborn pups were kept in a nested box with the dam until used. [*adenylate*-^{32}P]NAD$^+$ (20–22 Ci/mmol) and [α-^{32}P]ATP (20–35 Ci/mmol) were purchased from NEN Research Products, Boston, MA, U.S.A. Cholera toxin was purchased from Sigma Chemical Co. Pertussis toxin was procured from List Biological Laboratories, Campbell, CA, U.S.A. S49 cyc$^-$ cells were obtained from the Cell Culture Facility of the University of California, San Francisco. Plasma membranes were prepared from these cells by the method of Ross *et al.* (1977). The S49 cyc$^-$ membranes used in early stages of this study were kindly provided by Dr. Craig C. Malbon, State University of New York at Stony Brook, Stony Brook, NY, U.S.A. A synthetic peptide corresponding to amino acids 28–42 of α_s with a cysteine residue at the *N*-terminus (Mumby *et al.*, 1986) was purchased (catalogue no. 7701) from Peninsula Laboratories, Belmont, CA, U.S.A., and used as the source of antigen for generating anti-α_s antibodies. All other chemicals were obtained from readily available commercial sources.

Abbreviations used: G-protein, guanine-nucleotide-binding regulatory protein; G_s, the stimulatory G-protein; G_i, the inhibitory G-protein; G_o, the other G-protein; α_s and α_i, α-subunit of G_s and G_i; $\alpha_{s\text{-}1}$ and $\alpha_{s\text{-}2}$, subspecies of α_s; $\alpha_{i\text{-}1}$, $\alpha_{i\text{-}2}$ and $\alpha_{i\text{-}3}$, subspecies of α_i; cyc$^-$, G_s-deficient variant of the S49 lymphoma cell; TED, 20 mM-Tris/HCl, 1 mM-EDTA, 1 mM-dithiothreitol.

* To whom reprint requests should be addressed.

Preparation of liver plasma membranes and [^{32}P]ADP-ribosylation of membrane proteins

Partially purified liver membranes were prepared by sucrose-density-gradient centrifugation as described previously (Kawai *et al.*, 1985) and stored frozen at −80 °C until used. Protein in membranes was determined by the method of Lowry *et al.* (1951), with BSA as a standard, after membrane fractions were solubilized (Kawai *et al.*, 1985).

[^{32}P]ADP-ribosylation of membranes by cholera and pertussis toxins and quantification of ^{32}P-labelled proteins have been described previously (Kawai *et al.*, 1986). $NADP^+$ (0.75 mM) was included in all assay tubes to ensure maximal ADP-ribosylation of membrane proteins by both toxins (Kawai *et al.*, 1986).

Purification of G-proteins

G_i, G_o and their subunits were purified from bovine brain by the procedure of Sternweis & Robishaw (1984), except that octyl-Sepharose (Pharmacia) was used instead of heptylamine-Sepharose (Milligan & Klee, 1985). The partially purified G-proteins from the octyl-Sepharose column were then applied to a Mono Q HR5/5 column (Pharmacia) which had been equilibrated with 0.5% (w/v) Lubrol PX/TED, pH 7.6, and the column was eluted with a linear gradient of NaCl (0–400 mM) by using a Pharmacia f.p.l.c. apparatus. Major protein peaks were pooled, and each peak was re-chromatographed on the Mono Q column under the same conditions. The purified proteins, G_i, G_o and the $\beta\gamma$-subunit were identified by guanosine 5′-[γ-[^{35}S]thio]triphosphate-binding activity (Sternweis & Robishaw, 1984), pertussis-toxin-dependent [^{32}P]ADP-ribosylation and SDS/PAGE on a 10%-acrylamide gel by using a Mini-Protean II cell (Bio-Rad). These proteins were highly purified, as judged by silver staining of SDS/PAGE gels (Fig. 2*a*). The presence of the γ-subunit band (10 kDa) was evident in all three preparations, as analysed by SDS/PAGE in a 14%-acrylamide gel. G_s was partially purified from bovine liver by the same procedures as described above, except that after the first Mono Q column G_s-activity-containing fractions were applied to a Bio-Rad HPHT column fitted to the f.p.l.c. system (Graziano *et al.*, 1989) to remove contaminating G_i.

To determine the G-protein subunit concentrations in purified samples, proteins were separated by SDS/PAGE and electrophoretically transferred to nitrocellulose by using a Bio-Rad Trans-Blot apparatus. The nitrocellulose was washed with 10% trichloroacetic acid and stained with Amido Black by the procedure of Schaffner & Weissmann (1973). The concentrations of G-protein subunits were quantified by densitometry with BSA as a standard.

Preparation of antisera and affinity-purified antibodies

The synthetic α_s peptide (1 mg) was conjugated via its *N*-terminal cysteine to keyhole-limpet haemocyanin as described by Green *et al.* (1982). New Zealand White rabbits were injected intradermally with this conjugate (~ 400 μg per animal) or with purified G_i, G_o or $\beta\gamma$-subunit (100 μg per animal) in complete Freund's adjuvant, followed by two subsequent injections, at 2-week intervals, with the same amount of the conjugate or with half as much purified protein in incomplete adjuvant. Three rabbits were immunized with the conjugate; two rabbits were immunized with each purified G-protein. Animals were bled weekly; the antisera collected were subjected to heat inactivation at 56 °C for 10 min.

Antisera raised against the α_s-peptide conjugate were precipitated with 40%-satd. $(NH_4)_2SO_4$ and applied to an affinity column containing α_s-peptide-coupled Sepharose, which was prepared by coupling of the synthetic α_s peptide to activated CH-Sepharose 4B (Pharmacia). After extensive washing of unbound proteins, specific antibodies were eluted with 0.2 M-glycine/HCl (pH 2.5) and the eluate was immediately neutralized. This anti-peptide antibody recognizes the short form (45 kDa, i.e. $\alpha_{s\text{-}1}$) as well as the long form (52 kDa, i.e. $\alpha_{s\text{-}2}$) of α_s.

Immunoblot analysis

Liver plasma membranes (10–25 μg of protein) or purified G-proteins (10–40 ng) were subjected to SDS/PAGE on a 10%-acrylamide gel and electrophoretically transferred to nitrocellulose. The nitrocellulose was incubated with blocking solution containing 3% gelatin, followed by diluted rabbit antisera or affinity-purified antibodies, and then immunostained with alkaline-phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad) as a second antibody. Immunostained bands were scanned with an LKB laser densitometer, and the amounts of G-protein subunits in membranes were quantified by using purified G-proteins as standards.

G_s activity measured by cyc$^-$ reconstitution assays

Liver plasma membranes were extracted with 1% (w/v) sodium cholate/TED, pH 8.0, at a protein concentration of 10 mg/ml, and cholate extracts obtained after centrifugation (5 μg of protein) were reconstituted with cyc$^-$ membranes (40 μg of protein) as described by Sternweis *et al.* (1981). NaF-stimulated adenylate cyclase activity was then assayed as described previously (Kawai *et al.*, 1985). Under the reconstitution conditions the enzyme activity was proportional to the amounts of extracts added.

RESULTS

Development-dependent [^{32}P]ADP-ribosylation of cholera-toxin and pertussis-toxin substrates

In a previous study (Kawai *et al.*, 1985) we showed that the coupling efficiency in the β-adrenergic-receptor–adenylate cyclase system in rabbit liver is altered during neonatal development such that the system is less coupled or uncoupled at 0–6 h after birth and gradually becomes coupled after 6 h *post partum*. The data suggested that development-dependent changes in the amount and/or properties of G_s and/or G_i may be responsible, in part, for the alterations observed. The α-subunits of G_s and G_i (α_s and α_i) are substrates for ADP-ribosylation by the bacterial toxins cholera toxin and pertussis toxin respectively (Gilman, 1987). Therefore we initially assessed the relative amounts of G_s and G_i in membranes from neonatal and adult animals by labelling membranes with [^{32}P]NAD^+ in the presence of cholera and pertussis toxins. Fig. 1 shows that there was very little ^{32}P incorporation into cholera-toxin-dependent bands in membranes isolated from term animals. Thereafter the two major cholera-toxin-dependent ADP-ribosylated proteins corresponding to 45 kDa (short form, $\alpha_{s\text{-}1}$) and 52 kDa (long form, $\alpha_{s\text{-}2}$) became gradually evident, reaching maximal levels in membranes from 4–6-week-old animals. In contrast, the pertussis-toxin substrate (a 40/41 kDa doublet) was evident even in membranes from term animals and in all age groups studied. In the experiment in Fig. 1, which was run with 100 μg of membrane protein, this doublet seemed to band together; in other experiments, where we used less protein (Kawai *et al.*, 1986), the two bands were clearly visible. On the basis of molecular cloning and immunological data (Jones & Reed, 1987; Katada *et al.*, 1987; Mumby *et al.*, 1988; Itoh *et al.*, 1988; Freissmuth *et al.*, 1989; Strathmann *et al.*, 1989) at least three forms ($\alpha_{i\text{-}1}$, $\alpha_{i\text{-}2}$ and $\alpha_{i\text{-}3}$) of α_i have been identified. Because $\alpha_{i\text{-}1}$ is present predominantly in brain, and

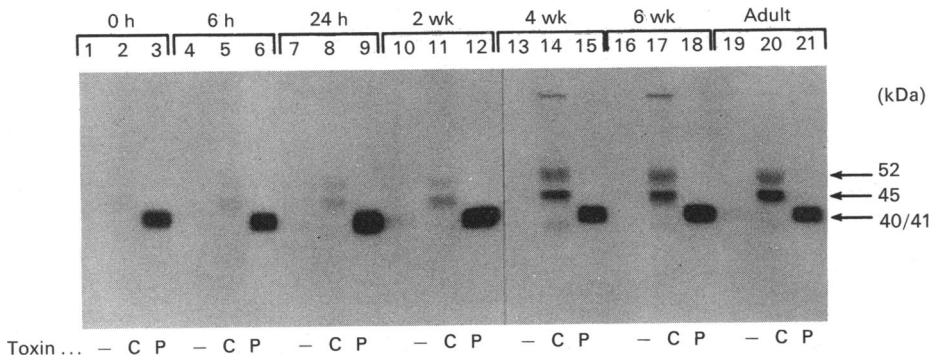

Fig. 1. Development-dependent [^{32}P]ADP-ribosylation of cholera-toxin and pertussis-toxin substrates in rabbit liver membranes

Membranes (100 μg of protein) from various age groups of animals were ADP-ribosylated in the absence (–) or presence of cholera toxin (C) or pertussis toxin (P) as described in the Experimental section. [^{32}P]ADP-ribosylated proteins were separated by SDS/PAGE on a 10%-acrylamide gel and detected by autoradiography. A representative autoradiogram is shown.

Table 1. Densitometric analysis of cholera-toxin- and pertussis-toxin-labelled bands in liver membranes from various age groups of rabbits

Membranes from various age groups of animals were ADP-ribosylated as described in the legend to Fig. 1. Autoradiograms were scanned and analysed with an LKB laser densitometer. Results are means ± S.E.M. for six different membrane preparations: *significantly different from corresponding adult values ($P < 0.05$, Mann–Whitney test).

	ADP-ribosylation (pmol/mg of protein)		
	G_s		G_i
Age of animals	45 kDa	52 kDa	40/41 kDa
0 h	0.13 ± 0.02*	0.09 ± 0.03*	1.51 ± 0.41*
6 h	0.21 ± 0.03*	0.15 ± 0.03*	1.75 ± 0.39*
24 h	0.22 ± 0.04*	0.17 ± 0.03*	3.09 ± 0.65
2 weeks	0.50 ± 0.08*	0.24 ± 0.05*	3.88 ± 0.46
4 weeks	1.06 ± 0.29	0.61 ± 0.17	2.97 ± 0.22
6 weeks	1.48 ± 0.28	0.77 ± 0.15	3.37 ± 0.31
Adult	1.75 ± 0.34	0.90 ± 0.10	3.17 ± 0.34

because little of it occurs in liver, which contains mostly $\alpha_{i\text{-}2}$ and $\alpha_{i\text{-}3}$ (Mumby *et al.*, 1988; Freissmuth *et al.*, 1989), the two bands detected by pertussis-toxin-dependent ADP-ribosylation in Fig. 1 would correspond to $\alpha_{i\text{-}2}$ (40 kDa) and $\alpha_{i\text{-}3}$ (41 kDa).

Densitometric analysis of the data in Fig. 1 showed that membranes isolated from term animals contained only 8% of the amount of α_s (45 kDa + 52 kDa proteins) and about 50% of that of α_i detected in membranes from the adult (Table 1). The pattern for α_s did not change for up to 24 h after birth; at 24 h the amounts of the two α_i bands already equalled adult values. When membranes from newborn animals were mixed with membranes from the adult, the extent of labelling of either α_s or α_i in the mixed samples was additive, suggesting the absence of inhibitor(s) or no influence from potential endogenous activator(s) for the ADP-ribosylation reaction in either set of membranes. Increased amounts of both cholera and pertussis toxins or higher concentrations of $NADP^+$ did not change the ADP-ribosylation patterns from those shown in Table 1.

Table 1 shows that alterations in the amounts of the two α_i subspecies during neonatal development were not as marked as for the subspecies of α_s. For example, there was no significant change in the α_i components after 1 day of life. A caveat, however, is that the availability of β- and γ-subunits during the period studied can influence the results obtained for this subunit, since the presence of $\beta\gamma$ is required to ADP-ribosylate the α-subunit of G_i (Tsai *et al.*, 1984). Conceivably the amount of α_i could increase, but if $\beta\gamma$ did not keep pace, the incorporation of [^{32}P]ADP-ribose into α_i would be constant. To test this possibility, membranes from newborn and adult animals were incubated with purified $\beta\gamma$-subunits and then ADP-ribosylated with pertussis toxin. The results did not show any significant differences in the developmental pattern of ^{32}P-labelling of α_i (results not shown), suggesting that the amount of $\beta\gamma$ in the membranes was probably not limiting for the ADP-ribosylation of α_i.

Use of antibodies to quantify G-protein subunits

Because cholera-toxin-catalysed ADP-ribosylation of α_s requires endogenous protein factors (Enomoto & Gill, 1980; Kahn *et al.*, 1988; Bobak *et al.*, 1989), the use of the ADP-ribosylation reaction to estimate the amounts of α_s in membranes may be problematical. In order to provide more sensitive and quantitative information on the G-protein subunits, we developed several polyclonal antibodies against a synthetic α_s peptide coupled to keyhole-limpet haemocyanin, as well as to purified G_i, G_o and the $\beta\gamma$-subunit. All rabbits injected produced anti-peptide antibodies with various yields and specificity as determined by immunoblot analysis. Antisera raised against the synthetic α_s-peptide conjugate reacted non-specifically with many proteins in liver membranes; therefore specific antibodies to α_s were affinity-purified from the antisera as described in the Experimental section. This affinity-purified antibody (Ab9) reacted only with $\alpha_{s\text{-}1}$ and $\alpha_{s\text{-}2}$ (see Fig. 2*b*).

Fig. 2 shows the specificity and reactivity of the affinity-purified antibody as well as some of the antisera used in this study. Antiserum As1, produced against the $\beta\gamma$-subunit, recognized primarily the β-subunit in purified G-proteins and liver membrane preparations (Fig. 2*c*), but did not distinguish between the 35- and 36-kDa subspecies of the β-subunit. Antisera As2 and As3 were raised against G_i and G_o respectively; As2 recognized α_i as well as the β-subunit (Fig. 2*d*) and As3 showed very high affinity to the β-subunit (Fig. 2*e*). The anti-G_i antiserum As2 was raised against purified G_i from bovine brain, which contains $\alpha_{i\text{-}1}$ and $\alpha_{i\text{-}2}$ (Mumby *et al.*, 1988). However, since we used whole G_i protein to produce As2, it is likely that this antiserum can readily cross-react with the major α_i subspecies in liver membranes, i.e. $\alpha_{i\text{-}2}$ and $\alpha_{i\text{-}3}$. Therefore these subspecies cannot be distinguished in our experiment. The anti-G_o antiserum As3 recognized only the β-subunit in liver membranes (Fig. 2*e*), but reacted strongly with α_o in bovine brain membranes (results

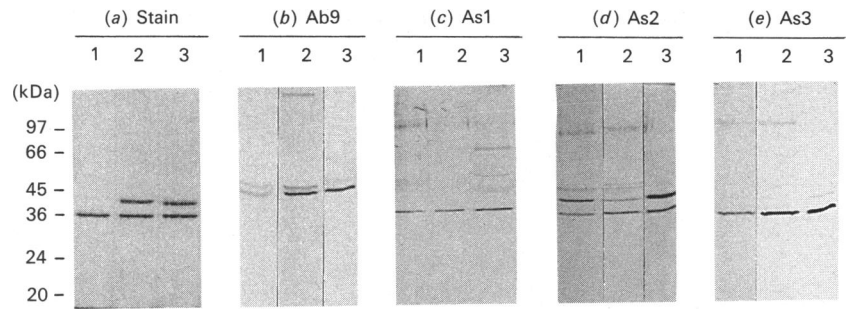

Fig. 2. Specificity of antibodies to G-protein subunits

Purified G-proteins and liver membranes were separated by SDS/PAGE on a 10%-acrylamide gel and either stained with silver (*a*) or transferred to nitrocellulose (*b–e*). The transblots were incubated with the indicated antibodies and immunostained with alkaline-phosphatase-conjugated goat anti-rabbit IgG as described in the Experimental section. (*a*) Silver-stained gel of purified G-proteins. Lanes were loaded with 0.15 μg of the $\beta\gamma$-subunit (1), 0.25 μg of G_i (2) and 0.25 μg of G_o (3). (*b*) Affinity-purified anti-α_s antibody Ab9, diluted 1/1000. Lanes were loaded with 25 μg each of membranes from 0 h-old newborn (1) and adult (2), and partially purified G_s containing 40 ng of α_s (3). (*c*) Anti-$\beta\gamma$-subunit antiserum As1, diluted 1/250. Lanes were loaded with 20 ng of $\beta\gamma$-subunit (1), 40 ng of G_i (2), and 25 μg of adult membranes (3). (*d*) Anti-G_i antiserum As2, diluted 1/250. Lanes were loaded with 20 μg each of membranes from 24 h-old newborn (1) and adult (2), and 40 ng of G_i (3). (*e*) Anti-G_o antiserum As3, diluted 1/250. Lanes were loaded with 10 μg each of membranes from 0 h-old newborn (1) and adult (2), and 40 ng of G_i (3). Values in the left-hand margin are for molecular-mass standards.

Table 2. Western-blotting analysis of concentration of G-protein subunits in liver membranes during neonatal development

Liver plasma membranes from various age groups of rabbits (10–25 μg of protein) were separated by SDS/PAGE and immunoblotted as described in the legend to Fig. 2. The immunostained G-protein bands were quantified densitometrically, with the purified G-proteins as a standard. Antibodies used were Ab9 for α_s, As2 for α_i, and As1 or As3 for the β-subunit. Results are means ± S.E.M. for four different membrane preparations for each age group: *significantly different from corresponding adult values ($P < 0.05$, Mann–Whitney test).

Age of animals	Subunit concn. (pmol/mg of protein)			
	$\alpha_{s\text{-}1}$	$\alpha_{s\text{-}2}$	α_i	β
0 h	6.1 ± 0.8*	2.7 ± 0.4*	12.3 ± 3.0	14.7 ± 1.3*
6 h	5.8 ± 0.5*	1.8 ± 0.3*	13.3 ± 3.6	13.5 ± 1.3*
24 h	6.9 ± 0.6*	3.2 ± 0.3*	22.4 ± 4.3	21.3 ± 6.1*
2 weeks	15.4 ± 0.5*	5.8 ± 0.9*	24.2 ± 4.4	36.7 ± 6.2
4 weeks	18.7 ± 1.0*	6.7 ± 0.4*	19.0 ± 2.2	43.7 ± 1.6
6 weeks	22.1 ± 1.3	10.5 ± 0.7	14.3 ± 1.6	48.1 ± 1.6
Adult	21.9 ± 0.5	10.2 ± 0.1	17.3 ± 2.4	46.7 ± 1.1

Table 3. G_s activity in rabbit liver membranes

Cholate extracts (5 μg of protein) of membranes from various age groups of animals were reconstituted with cyc$^-$ membranes (40 μg of protein) at 30 °C for 10 min. The concentration of cholate in the reconstitution phase was 0.1%. Adenylate cyclase activity in the mixture was determined in the presence of 10 mM-NaF. Results are means ± S.E.M. for two different liver membrane preparations. NaF-stimulated cyclase activity in cyc$^-$ membranes in the absence of cholate extracts was 0.03 ± 0.00 pmol/min.

Age of animals	Adenylate cyclase activity (pmol of cyclic AMP/min)
0 h	0.86 ± 0.08
6 h	0.94 ± 0.05
12 h	1.18 ± 0.08
24 h	1.24 ± 0.00
2 weeks	1.05 ± 0.10
4 weeks	1.57 ± 0.32
6 weeks	2.05 ± 0.22
Adult	2.19 ± 0.34

not shown); liver probably does not express G_o (Gilman, 1987; Mumby *et al.*, 1988).

Using the antibodies described above, we quantified the concentrations of G-protein subunits in hepatic plasma membranes isolated from various age groups of animals. Table 2 shows that the amounts of $\alpha_{s\text{-}1}$ and $\alpha_{s\text{-}2}$ did not change during 0–24 h after birth. Thereafter gradual increases occurred, from initial values of 6.1 ± 0.8 and 2.7 ± 0.4 pmol/mg of protein to maximal levels of 22.1 ± 1.3 and 10.5 ± 0.7 pmol/mg of protein for $\alpha_{s\text{-}1}$ and $\alpha_{s\text{-}2}$ respectively, within 6 weeks. The 6-week values were similar to those found in membranes from adult animals. The ratio of $\alpha_{s\text{-}2}$ to $\alpha_{s\text{-}1}$ was about 0.4 throughout all age groups. Unlike α_s, the amount of α_i was not dramatically different between the term newborn (12.3 ± 3.0 pmol/mg of protein) and the adult (17.3 ± 2.4 pmol/mg of protein), but a transient increase was noticeable in 24 h- and 2-week-old animals. This pattern paralleled that determined by ADP-ribosylation assay (cf. Fig. 1 and Table 1). The level of the β-subunit in membranes from 0–6 h-old animals was only 14.7 ± 1.3 and 13.5 ± 1.3 pmol/mg of protein for term and 6 h-old animals respectively, but it gradually increased to the adult value of 46.7 ± 1.1 pmol/mg of protein within 6 weeks. Since the antisera used to assess the β-subunit probably recognized the two forms of the β-subunit, it can be concluded from Table 2 that the developmental profile of the β-subunit almost mimicked that of the G_s subunits.

G_s activity during neonatal development

Because the most dramatic developmental change occurred in the G_s component, we were interested in correlating changes in this component with the function of G_s in liver membranes as measured by G_s activity, via reconstitution with cyc$^-$ membranes. It is generally accepted that G_s activity determined by reconstitution assay using cyc$^-$ membranes is a measurement of G_s function, and that it reflects directly the amount of G_s present in the test systems (Sternweis & Gilman, 1979; Codina *et al.*, 1985). Table 3 shows that the G_s activity in cholate extracts of membranes from term animals is about 40% of maximal values; the activity at term was relatively constant in the first 6 h after birth, but increased gradually with age of the animals thereafter. Its developmental pattern is similar to that of the concentrations

of the α_s subspecies determined by Western blotting (Table 2). This pattern was also similar to that of the cholera-toxin-dependent ^{32}P-labelling of the α_s components in membranes (cf. Fig. 1 and Table 1), although the magnitude of the changes was somewhat different from those for ^{32}P-labelling.

DISCUSSION

We were interested in the potential physiological impact of the changes in the concentrations of G-protein subunit during development. The G_s activity (Table 3), which represents a functional measurement for any G_s present (Sternweis & Gilman, 1979; Codina *et al.*, 1985), parallels that of the amount of G_s determined by immunoblot analysis (Table 2), indicating that functionally active G_s is increasing during neonatal development. We previously reported that stimulation of adenylate cyclase by β-agonists in rabbit liver membranes is least at 4–6 h after birth, occurring at only one-quarter of the adult value, even though the density of β-adrenergic receptors remains unaltered within the range of 200–400 fmol/mg of protein and there are no apparent quantitative changes in the catalytic unit of adenylate cyclase during neonatal development (Kawai *et al.*, 1985). Comparing the values for β-receptors with the amounts of α_s determined by immunoblot analysis in this study, one can calculate ratios of total α_s to the β-receptor of 30 and 110 for membranes from the newborn and adult respectively. If the function of G_s in liver membranes correlates with the amount of G_s present, as described above, it can be speculated that, even considering the presence of other types of receptors coupled to adenylate cyclase through G_s, a considerably high excess of G_s over β-receptors is required to activate adenylate cyclase fully in this system. S49 lymphoma cells, for example, also contain more than a 100-fold excess of α_s over β-receptors (Ransnäs & Insel, 1988).

The experimental approach used in the present study was to quantify at the protein level the concentrations of the subunits of heterotrimeric G-proteins, specifically G_s and G_i, in rabbit liver plasma membranes during neonatal development. By use of immunoblotting methods, the results demonstrate 3–4-fold increases in levels of the β-subunit as well as the short ($\alpha_{s\text{-}1}$) and long ($\alpha_{s\text{-}2}$) forms of α_s, and a transient increase in two forms of α_i, with age of the animals. The concentrations measured with this technique are much higher (more than 10 times for α_s and 5–10 times for α_i) than those determined by ADP-ribosylation assay (Table 1 versus Table 2), a phenomenon consistent with the general conclusion by others (Watkins *et al.*, 1987; Ransnäs & Insel, 1988) that the ADP-ribosylation assay may underestimate the concentrations of G-proteins. However, by both methods the developmental pattern of G-proteins measured in this study is similar, suggesting that the ADP-ribosylation technique can be used to detect the relative abundance of G-proteins. Ransnäs & Insel (1988) reported that the concentration of G_s in purified membranes from various mutant S49 lymphoma-cell lines measured by an antibody-based technique was about 20 pmol/mg of protein, a value comparable with our data. Because the γ-subunit was not quantified in our studies, we cannot draw conclusions regarding this subunit.

Since it is believed that dissociation of $\beta\gamma$-subunits of G_s or G_i complex results in stimulation or inhibition of adenylate cyclase (Gilman, 1987; Birnbaumer *et al.*, 1987; Neer & Clapham, 1988), it is intriguing to speculate that the formation and dissociation of G_s complexes in test membranes, *in situ*, can be greatly influenced by competition for β- and/or γ-subunit present. This is because the β- and γ-subunits are presumed to be common to G_s and G_i complexes (Codina *et al.*, 1986; Fong *et al.*, 1986; Gilman, 1987), although subsequent studies using molecular-cloning methods and subspecies-specific antibodies have revealed heterogeneity even in the β (Woolkalis & Manning, 1987; Amatruda *et al.*, 1988) and γ (Robishaw *et al.*, 1989) components. On the basis of our immunoblotting data, however, the concentration of the β-subunit, at any of the age groups studied, was not greater than the sum of the α-subunits, suggesting that there may be little or no free $\beta\gamma$-subunit in this system, although it can be argued that antibodies raised against bovine brain proteins may not be absolute probes for rabbit liver proteins.

Molecular-cloning methods have shown that G_s and G_i are programmed by different genes (Ashley *et al.*, 1987; Blatt *et al.*, 1988). Regulatory processes governing the expression of these genes are of current interest. By using molecular probes as well as antibodies, Luetje *et al.* (1988) have shown differences in the expression of G_i and G_o and their subunits in rat atria and ventricles during cardiac development. Our studies provide a groundwork for further analysis of the genetic expression of G-protein subunits during neonatal development in mammalian liver. It would be of interest to determine specifically which subspecies of the G-protein subunits is primarily associated with a given metabolic process.

We thank Gale Beamer and Jacqueline Harding for typing the manuscript, and Dr. Craig C. Malbon, Department of Pharmacological Sciences, State University of New York at Stony Brook, for providing the S49 cyc$^-$ membranes used in initial studies. This work was supported by grant HD 08792 from the National Institutes of Health, and by grant 1-598 from the March of Dimes–Birth Defects Foundation.

REFERENCES

Amatruda, T. T., III, Gautam, N., Fong, H. K. W., Northup, J. K. & Simon, M. I. (1988) J. Biol. Chem. **263**, 5008–5011
Ashley, P. L., Ellison, J., Sullivan, K. A., Bourne, H. R. & Cox, D. R. (1987) J. Biol. Chem. **262**, 15299–15301
Birnbaumer, L., Codina, J., Mattera, R., Yatani, A., Scherer, N., Toro, M.-J. & Brown, A. M. (1987) Kidney Int. Suppl. **23**, S14–S37
Blatt, C., Eversole-Cire, P., Chohn, V. H., Zollman, S., Fournier, R. E. K., Mohandas, L. T., Nesbitt, M., Lugo, T., Jones, D. T., Reed, R. R., Weiner, L. P., Sparkes, R. S. & Simon, M. I. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 7642–7646
Bobak, D. A., Nightingale, M. S., Murtagh, J. J., Price, S. R., Moss, J. & Vaughan, M. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 6101–6105
Codina, J., Rosenthal, W., Hildebrandt, J. D., Birnbaumer, L. & Sekura, R. D. (1985) Methods Enzymol. **109**, 446–465
Codina, J., Stengel, D., Woo, S. L. C. & Birnbaumer, L. (1986) FEBS Lett. **207**, 187–192
Enomoto, K. & Gill, D. M. (1980) J. Biol. Chem. **255**, 1252–1258
Fong, H. K. W., Hurley, J. B., Hopkins, R. S., Miake-Lye, R., Johnson, M. S., Doolittle, R. F. & Simon, M. I. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 2162–2166
Freissmuth, M., Casey, P. J. & Gilman, A. G. (1989) FASEB J. **3**, 2125–2131
Gilman, A. G. (1987) Annu. Rev. Biochem. **56**, 615–649
Graziano, M. P., Freissmuth, M. & Gilman, A. G. (1989) J. Biol. Chem. **264**, 409–418
Green, N., Alexander, H., Olson, A., Alexander, S., Schinnick, T. M., Sutcliffe, J. G. & Lerner, R. A. (1982) Cell **28**, 477–487
Itoh, H., Toyama, R., Kozasa, T., Tsukamoto, T., Matsuoka, M. & Kaziro, Y. (1988) J. Biol. Chem. **263**, 6656–6664
Jones, D. T. & Reed, R. R. (1987) J. Biol. Chem. **262**, 14241–14249
Kahn, R. A., Goddard, C. & Newkirk, M. (1988) J. Biol. Chem. **263**, 8282–8287
Katada, T., Oinuma, M., Kusakabe, K. & Ui, M. (1987) FEBS Lett. **213**, 353–358
Kawai, Y., Graham, S. M., Whitsel, C. & Arinze, I. J. (1985) J. Biol. Chem. **260**, 10826–10832
Kawai, Y., Whitsel, C. & Arinze, I. J. (1986) J. Cyclic Nucleotide Protein Phosphorylation Res. **11**, 265–274
Lang, J. (1989) Eur. J. Biochem. **183**, 687–692
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
Luetje, C. W., Tietje, K. M., Christian, J. L. & Nathanson, N. M. (1988) J. Biol. Chem. **263**, 13357–13365

Milligan, G. & Klee, W. A. (1985) J. Biol. Chem. **260**, 2057–2063
Mumby, S. M., Kahn, R. A., Manning, D. R. & Gilman, A. G. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 265–269
Mumby, S., Pang, I.-H., Gilman, A. G. & Sternweis, P. C. (1988) J. Biol. Chem. **263**, 2020–2026
Neer, E. J. & Clapham, D. E. (1988) Nature (London) **333**, 129–134
Ransnäs, L. A. & Insel, P. A. (1988) J. Biol. Chem. **263**, 9482–9485
Robishaw, J. D., Kalman, V. K., Moomaw, C. R. & Slaughter, C. A. (1989) J. Biol. Chem. **264**, 15758–15761
Ross, E. M., Maguire, M. E., Sturgill, T. W., Biltonen, R. L. & Gilman, A. G. (1977) J. Biol. Chem. **252**, 5761–5775
Schaffner, W. & Weissmann, C. (1973) Anal. Biochem. **56**, 502–514
Skorecki, K. L., Verkman, A. S. & Ausiello, D. A. (1987) Biochemistry **26**, 639–645
Sternweis, P. C. & Gilman, A. G. (1979) J. Biol. Chem. **254**, 3333–3340
Sternweis, P. C. & Robishaw, J. D. (1984) J. Biol. Chem. **259**, 13806–13813
Sternweis, P. C., Northup, J. K., Smigel, M. D. & Gilman, A. G. (1981) J. Biol. Chem. **256**, 11517–11526
Strathmann, M., Wilkie, T. M. & Simon, M. I. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 7407–7409
Tsai, S.-C., Adamik, R., Kanaho, Y., Hewlett, E. L. & Moss, J. (1984) J. Biol. Chem. **259**, 15320–15323
Watkins, D. C., Northup, J. K. & Malbon, C. C. (1987) J. Biol. Chem. **262**, 10651–10657
Watkins, D. C., Northup, J. K. & Malbon, C. C. (1989) J. Biol. Chem. **264**, 4186–4194
Woolkalis, M. J. & Manning, D. R. (1987) Mol. Pharmacol. **32**, 1–6

Received 26 July 1990/24 October 1990; accepted 2 November 1990