Levels of G-proteins in liver and brain of lean and obese (*ob/ob*) mice

Norma McFARLANE-ANDERSON, Jane BAILLY and Nicole BÉGIN-HEICK*

Department of Biochemistry, University of Ottawa, Ottawa, Ont. K1H 8M5, Canada

G-protein levels were assessed in liver and brain membranes of lean and obese mice. ADP-ribosylation and immunodetection studies revealed a decrease in the abundance of G_s and G_i α -subunits in the liver membranes of obese mice compared with lean mice. In contrast, in brain membranes, the abundance of these proteins was not significantly different between lean and obese mice. Studies at the mRNA level in both liver and brain revealed no difference in gene expression between lean and obese mice. Protein and mRNA studies both showed that G_s , G_{i1} , G_{i2} , $G_o\alpha$ and $G\beta$ subunits are present in brain membranes, and G_{i3} is barely detectable. In liver, $G_s\alpha$, G_{i2} and $G\beta$ subunits are the major constituents, whereas G_{i1} , G_{i3} and $G_o\alpha$ are barely detectable. It is possible that the differences observed at the protein level are due to different rates of translation of the mRNA. Different rates of release of the α -subunits from the membrane and/or different rates of degradation would also explain these results.

INTRODUCTION

The transducing element of adenylate cyclase (AC) is composed of two sets of heterotrimeric proteins, G_s and G_i [see Gilman (1987) and references therein]. G_s mediates the activation of the catalytic unit of AC, whereas G_i prevents it. Cells may contain up to four different isoforms of $G_s\alpha$ (α subunit of G_s) with relative mobilities corresponding to approx. 46 and 42 kDa produced by differential splicing of a common mRNA precursor (Robishaw *et al.*, 1986; Mattera *et al.*, 1986). These are substrates for ADP-ribosylation by cholera toxin. Each of the four variants of $G_s\alpha$ has been found to stimulate AC and Ca^{2+} channels (Mattera *et al.*, 1989). Several pertussis-toxin substrates ranging from 39 to 41 kDa have also been discovered by cDNA cloning (Suki *et al.*, 1987; Jones & Reed, 1987): three of these have been classified as G_{i1} , and one as G_o . Immunodetection with specific antibodies has allowed the identification of these proteins in various tissues and shows a tissue-selective expression of the various forms (Goldsmith *et al.*, 1987, 1988; Mitchell *et al.*, 1989; Simonds *et al.*, 1989). The exact function of the individual pertussis-toxin substrates is not yet known with certainty, but G_o is thought to be involved in the mediation of Ca^{2+} -channel activity (Goldsmith *et al.*, 1988), G_{i3} is able to regulate K^+ channels (Codina *et al.*, 1988) and G_{i2} mediates the α_2 -adrenergic inhibition of AC (Simonds *et al.*, 1989).

A large compendium of data has led to the presently held view that the dual regulation of AC is the result of the mass-action effect of the $\beta\gamma$ subunit. According to the model, although $G_s\alpha$ is an activator of the catalytic unit of AC, the main function of the α -subunit of G_i is to act as a receptacle for the $\beta\gamma$ subunits (Gilman, 1987). Although the model does not explain all of the findings, it is widely used to explain AC regulation. Many mutants of the AC system have been found (Haga *et al.*, 1977; Bourne *et al.*, 1982; Schimmer & Tsao, 1984) which alter the regulatory properties of AC. There are several examples showing that tissues differ in the proportion of regulatory components that they contain and that the relative amount of the subunits of the transducing G-proteins is subject to modulation (Bokoch, 1987; Carter *et al.*, 1987; Milligan *et al.*, 1987; Watkins *et al.*,

1987; Hinsch *et al.*, 1988; Houslay *et al.*, 1989; Garcia-Sainz *et al.*, 1989; Green & Johnson, 1989; Rapiejko *et al.*, 1989).

We have recently demonstrated that adipocyte membranes of lean and obese mice differ in the pattern of the G proteins they contain (Bégin-Heick, 1990). Also the ability of AC to be regulated by ligands is altered in adipocyte (Bégin-Heick, 1985; Bégin-Heick & Coleman, 1988) and liver (Bégin-Heick & Welsh, 1988) membranes of obese compared with lean mice. The present work was therefore designed to answer the following questions. (a) Is this altered G-protein pattern also found in other tissues of the *ob/ob* mouse? (b) Is it a result of the differential expression of the mRNA that encodes these proteins? Since anomalous AC activity could result from excess β -subunit, which could be achieved by overproduction of the β -subunit, decreased levels of G_s or increased levels of G_{i1} , particular attention was paid to these subunits. The data presented in this paper show that, compared with those of the lean mouse, liver membranes (like adipocyte membranes) from the *ob/ob* mouse have a lower abundance of G_{i2} and $G_s\alpha$ subunits. This is not the case in brain membranes, where there is no significant difference in the abundance of the $G_s\alpha$ subunits or of any of the subunits corresponding to the pertussis-toxin substrates.

EXPERIMENTAL

Materials

[α - ^{32}P]ATP, cyclic [3H]AMP, [3H]AMP and [^{32}P]NAD $^{+}$ were obtained from New England Nuclear Corp., Lachine, Que., Canada. [γ - ^{32}P]ATP, deoxycytidine 5'-[α - ^{32}P]triphosphate, ^{125}I -labelled goat anti-rabbit IgG, Hybond nylon membrane and the multi-prime labelling kit were from Amersham Canada Ltd., Oakville, Ont., Canada. Caffeine, ATP (cat. no. A-2383, prepared by phosphorylation of adenosine), cyclic AMP, phosphocreatine (Tris salt), creatine kinase, myokinase, L(–)-isoprenaline, salmon sperm DNA, dextran sulphate and cholera toxin were from Sigma Chemical Co., St. Louis, MO, U.S.A. GTP was from P-L Biochemicals, Milwaukee, WI, U.S.A. Pertussis toxin was from List Biochemicals (Campbell, CA, U.S.A.). Universol was from ICN Biomedicals Corp., Montreal, Que., Canada: NACS

Abbreviation used: AC, adenylate cyclase (EC 4.6.1.1).

* To whom correspondence should be addressed.

The cDNA sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X62634.

Prepac columns and the restriction enzymes were from GIBCO/BRL, Life Technologies Inc., Burlington, Ont., Canada. The mouse cDNA library was from Clontech Laboratories, Palo Alto, CA, U.S.A. The anti-G-protein antibodies (see Goldsmith *et al.*, 1988; Simonds *et al.*, 1989) were from NEN/DuPont Canada, Mississauga, Ont., Canada.

Animals

Male C57B1/6J *ob/ob* mice and lean controls (+/+) were obtained from Jackson Laboratories, Bar Harbor, ME, U.S.A., at 7–8 weeks of age. They were kept in a temperature-controlled room ($23 \pm 1^\circ\text{C}$) with 12 h-light/12 h-dark cycles. The animals were fed on Purina Chow and water *ad libitum*; they were used in the experiments at the age of 9–12 weeks.

Preparation of membranes

The mice were killed by cervical dislocation, and the liver and brain were removed and immediately frozen in liquid nitrogen and kept at -80°C , until used for the preparation of membranes. Liver membranes were prepared as described previously (Bégin-Heick & Welsh, 1988), except that they were washed in a buffer containing 10 mM-potassium phosphate, pH 7.4, 1 mM-EDTA and 1 mM-dithiothreitol (KPED buffer) and resuspended in KPED buffer containing 40 μM -leupeptin and 1 μg of trypsin inhibitor/ml (storage buffer). Frozen brains were homogenized by hand in a Potter–Elvehjem homogenizer, with 2 ml of 0.25 M-sucrose adjusted to pH 7.4 with 5 mM-Tris/HCl for each brain. The homogenate was centrifuged at 1500 *g* for 10 min. The supernatant fraction was collected and centrifuged for 15 min at 100000 *g* in the SW-28 rotor of the Beckman ultracentrifuge. The sediment was resuspended in the equivalent of 2.5 ml of KPED buffer/brain and the centrifugation was repeated. This washing procedure was repeated twice more. The final pellet was resuspended in the equivalent of 1 ml of the storage buffer/brain. The membranes were stored in small portions at -80°C , until used.

Enzyme assays

(a) **AC.** This was carried out essentially as described by Bégin-Heick (1985). The incubation mixture contained the following components in a total volume of 0.1 ml: 30 mM-Tris buffer containing 0.75 mM-EGTA adjusted to pH 7.4 with 0.2 M-acetic acid and 1 mM-MgSO₄; 20 mM-phosphocreatine, 1.75 units of creatine kinase, 1 unit of adenosine deaminase, 0.1 mM-cyclic AMP and 0.05 mM- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ (2.6×10^6 c.p.m./assay). Cyclic $[\text{H}^3]\text{AMP}$ (12000–15000 c.p.m./assay) was added to monitor the recovery of cyclic $[\text{H}^3]\text{AMP}$. For the assay of the enzyme in brain membranes, 0.3 mM-CaCl₂ was added and the EGTA concentration was lowered to 0.2 mM. Other components of the reaction were added as specified in the legends to Tables. After addition of enzyme (10 μg of protein), the tubes were incubated with shaking at 30°C for 10 min. The incubation was terminated by addition of 0.1 ml of a solution consisting of 0.2% SDS, 1.4 mM-cyclic AMP and 40 mM-ATP at pH 7.5, followed by 0.8 ml of water. Cyclic $[\text{H}^3]\text{AMP}$ was separated from the $[\text{H}^3]\text{ATP}$ as previously described (Bégin-Heick, 1985). The eluate from the alumina column was collected and counted for radioactivity in a Beckman LS-250 liquid-scintillation counter after addition of 10 ml of Universol.

(b) **5'-Nucleotidase (EC 3.1.3.5).** This was done essentially by the method of Avruch & Wallach (1971), using concentrations of AMP in the range 5–100 μM ; 2–5 μg of membrane protein was added to start the reaction, and the tubes were incubated for 30 min at 37°C .

(c) **Na⁺,K⁺-ATPase (EC 3.6.1.3).** This was also essentially by the method of Essman (1988), except that $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was used.

The concentrations of ATP ranged from 0.5 to 3 mM. The reaction was started by addition of 10 μg of membrane protein, and the tubes were incubated for 10 min at 37°C . The liberated P_i was separated from ATP by adsorption on charcoal as described by Avruch & Wallach (1971).

Activation of membranes with cholera toxin

This was done essentially by the method of Ribeiro-Neto *et al.* (1987). Specific modifications were introduced to maximize the ADP-ribosylation in each tissue, and the following were found to be essential for maximal incorporation of labelled ADP-ribose and maximal specificity of incorporation. For liver membranes, cholera toxin was pre-activated by incubation at 37°C for 10 min in a medium consisting of 0.01 M-Tris/HCl buffer, pH 7.5, 0.04 M-NaCl, 0.6 mM-NaN₃, 0.2 mM-Na₂EDTA, 50 mM-glycine and 20 mM-dithiothreitol. The membranes (100 μg of protein) were preincubated at 22°C for 10 min in 250 μl of a medium containing 10 μg of pre-activated cholera toxin, 20 mM-Tris/HCl, pH 7.5, 1 mM-GTP, 10 mM-MgSO₄, 1 mM EDTA, 300 mM-P_i, 10 mM-thymidine, 1 mM-nicotinamide, 10 mM-NADP⁺ and a regeneration system consisting of 20 mM-phosphocreatine, 40 units of creatine kinase/ml and 40 units of myokinase/ml. At the end of the preincubation, 4 μM - $[\text{H}^3]\text{NAD}^+$ (sp. radioactivity 15 $\mu\text{Ci}/\text{mmol}$) was added to start the reaction. The tubes were incubated at 30°C for 30 min. The reaction was stopped by addition of 4 ml of ice-cold Tris/HCl buffer (20 mM, pH 7.5), followed by centrifugation for 20 min at 15000 *g* to sediment the membranes. The membranes were washed twice with cold buffer and dissolved in electrophoresis buffer as described below. For brain membranes, the procedure was essentially the same, except that NADP⁺ was omitted from the activation medium and 20 mM-isonicotinic hydrazide was added instead. It was also necessary to add 0.025% SDS to the medium to keep the brain membranes dispersed during the ADP-ribosylation reaction.

Treatment with pertussis toxin

Pertussis toxin was pre-activated for 15 min at 30°C in a medium consisting of 20 mM-Tris/HCl, pH 7.5, 2 mM-EDTA, 1 mM-ATP, 20 mM-dithiothreitol and 1% Lubrol-PX. The membranes (50 μg of protein) were added to a medium consisting of 2 μg of the activated toxin, 20 mM-Tris/HCl, pH 7.5, 10 mM-thymidine, 1 mM-urea, 1 mM-ATP, 1 mM-EDTA, and the ATP-regenerating system described above for cholera-toxin activation. The mixture was preincubated at 22°C for 10 min, after which 5 μM - $[\text{H}^3]\text{NAD}^+$ (15 $\mu\text{Ci}/\text{mmol}$) was added to start the reaction and the tubes were incubated at 4°C for 180 min. The reaction was stopped by addition of NaCl and acetone, and the membranes were sedimented and extracted as described by Ribeiro-Neto *et al.* (1987). Brain membranes were ribosylated in the same manner, except that 1 mM-nicotinamide was present during the preincubation and the incubation.

SDS/PAGE

This was carried out in slab gels by standard methods. Essentially, a 3% (w/v)-acrylamide stacking gel and a 10% (w/v)-acrylamide separating gel were used, with a ratio of acrylamide to bisacrylamide of 75:1. Membrane suspensions were dissolved in an equal volume of sample buffer containing 2% SDS, 10% (v/v) 2-mercaptoethanol, 2 mM-EDTA, 4 mM-phenylmethanesulphonyl fluoride and 100 mM-Tris/HCl, pH 7.8, and boiled for 3 min before application to the gel. After electrophoresis, the gels were dried and autoradiographed to assess the extent of $[\text{H}^3]\text{ADP}$ -ribose incorporation. The intensity of the bands was assessed with a laser densitometer LK7 Ultrosan XL (LKB). To allow quantification of the intensity of the signal in terms of mol of ^{32}P incorporated, standards of

known concentrations containing known amounts of radioactivity were autoradiographed along with the gels. The intensity of the signal quantified by laser densitometry was used to construct standard curves, as described by Swillens *et al.* (1989).

Immunoblotting

After electrophoresis, the gels were soaked for 30 min in transfer buffer (25 mM-Tris, 192 mM-glycine, 20% methanol, pH 8.3). Proteins were transferred from the gels to presoaked nitrocellulose membranes at 300 mA for 2.5 h. The nitrocellulose membranes were stained with Ponceau Red (to ensure efficiency of transfer), washed in phosphate-buffered saline (137 mM-NaCl, 2.7 mM-KCl, 4.3 mM-sodium phosphate, 1.4 mM-potassium phosphate, pH 7.3) and either used immediately or stored at -20°C . Membranes were incubated for approx. 1 h in 10 mM-Tris/HCl (pH 7.5)/500 mM-NaCl containing 3% skim milk (buffer A).

Immunodetection was carried out by incubating the membrane with the specific antibody diluted 1:100 with buffer A, for 3 h at 37°C . The membranes were washed several times during 15–30 min with Tris/HCl/NaCl containing 0.2% Tween 20 (buffer B). Membranes were then incubated for 2 h at room temperature with a ^{125}I -labelled goat anti-rabbit IgG diluted 1:100 with buffer A. After extensive washing with several changes of buffer B, the membranes were dried and subjected to autoradiography. The intensity of the radioactive spots was assessed by densitometry. The data are expressed as arbitrary units, corrected for the protein concentration and the time of exposure by comparison with standards of known radioactivity, as described under SDS/PAGE. All assays were done at three different protein concentrations.

β -subunit cDNA isolation

Human liver β -subunit cDNA, consisting of 3088 bp (Codina *et al.*, 1986), was generously provided by Dr. J. Codina. An 830 bp fragment was used to screen a mouse cDNA library in λ gt11 (Clontech) and yielded, after tertiary screening, a 369 bp fragment, which was subcloned into pSP64 and sequenced by the dideoxynucleotide chemical termination method.

α -subunit probes

cDNAs for the α -subunits G_{i1} , G_{i2} , G_{i3} , G_o and G_s obtained by screening a rat olfactory cDNA library (Jones & Reed, 1987) were provided by Dr. R. R. Reed (Johns Hopkins University, Baltimore, MD, U.S.A.). Mouse G_s and G_{i2} cDNAs isolated from a mouse macrophage cDNA library (Sullivan *et al.*, 1986) were given by Dr. S. Masters (University of California, San Francisco, CA, U.S.A.). The mouse and rat cDNAs, which all contained the full-length coding sequences, were used to transform DH5 cells and the *Eco*R1 fragments isolated. Since there is a high degree of conservation of the G-protein α -subunits, rat as well as mouse cDNAs were used to probe Northern blots and dot-blots.

RNA preparation

Total RNA was isolated from brain and liver of lean (+/+) and obese (*ob/ob*) mice (either fresh tissue or tissue frozen in liquid nitrogen) by using LiCl/urea. Samples were size-fractionated by electrophoresis in 1.5%-agarose gels containing 6% (v/v) formaldehyde. The RNA was transferred to Hybond nylon membranes and subjected to Northern-blot analysis as described below. RNA samples (0.125–2.0 μg) denatured in 50% (v/v) formamide/6% (v/v) formaldehyde/20 mM-Mops/5 mM-sodium acetate/1 mM-EDTA (pH 7.0) solution were applied to Hybond membranes either manually or with a HYBRI-SLOT manifold (BRL) and hybridized as described below.

Hybridization

The mouse probe was labelled in a multi-prime DNA-labelling reaction using [^{32}P]dCTP (Amersham). Labelled DNA was eluted from NACS Prepac columns (BRL) with 2.0 M-NaCl/10 mM-Tris/HCl/1 mM-EDTA, pH 7.4. Hybridizations were carried out at 42°C overnight in 50% formamide/5 $\times$ standard saline phosphate containing 1 mM-EDTA (SSPE; 180 mM-NaCl/100 mM-sodium phosphate/1 mM-EDTA, pH 7.7)/10% (w/v) dextran sulphate/5 $\times$ Denhardt's solution/20 μg of salmon sperm DNA/ml. The filters were washed twice for 15 min in 2 $\times$ standard saline citrate (SSC; 150 mM-NaCl/15 mM-sodium citrate)/0.1% SDS at room temperature and for 15 min in 0.2 $\times$ SSC/0.1% SDS at 42°C , and then subjected to autoradiography. Autoradiograms were analysed by densitometry.

Protein determination

The protein content of the membrane fractions was determined by the method of Bradford (1974), with bovine serum albumin as the standard.

Statistical analysis

The difference between means was evaluated by Student's *t* test for matched pairs. The *P* values are given in each Table. Differences between means were considered to be statistically significant when *P* < 0.05.

RESULTS

Characterization of membranes

The activities of 5'-nucleotidase and Na^+, K^+ -ATPase were measured to assess whether the membranes prepared from each type of mice were comparable. The only difference observed between the two groups was that the V_{max} of Na^+, K^+ -ATPase for ATP was about 25% greater in brain membranes from obese than from lean mice (Table 1). The basal and maximal AC activities were not significantly different in the lean and obese groups, for either tissue (Table 1). This demonstrates that the membrane fractions are not differentially contaminated by other tissue fractions in tissues of lean or obese animals.

Measurement of G-proteins by ADP-ribosylation

(a) **Liver membranes.** The incorporation of ADP-ribose stimulated by cholera toxin in liver membranes of lean and obese mice is documented in Table 2 and Fig. 1. In membranes of obese mice compared with those of lean, the incorporation of ADP-ribose was significantly less in both the 42 and 46 kDa peptides, so that the total amount of ADP-ribose incorporated was only half as much in the obese as in the lean membranes, but the ratio of these peptides was the same in both membranes. Likewise, the incorporation of ADP-ribose stimulated by pertussis toxin was decreased by more than 40% in membranes of obese compared with lean mice (Table 3 and Fig. 1). The ratio of pertussis-toxin to cholera-toxin substrates in the liver membranes of lean and obese mice was therefore comparable (10:1 and 12:1 respectively).

(b) **Brain membranes.** There were no significant differences in the amount of ADP-ribose incorporation stimulated by cholera toxin in the brain membranes of lean or obese mice (Table 2 and Fig. 1). Whereas in other tissues (adipocytes, liver) examined, the ratio of 46 kDa/42 kDa peptide was approx. 1, in brain membranes the abundance of the long isoform (46 kDa) was more than 2.5 times that of the short isoform (42 kDa). There was no significant difference between lean- and obese-mouse tissues in the abundance of the peptides ADP-ribosylated by pertussis toxin (Table 3 and Fig. 1).

Table 1. Activities of marker enzymes in liver and brain membranes of lean (+/+) and obese (ob/ob) mice

The enzyme assays were done as described in the Experimental section. The data shown are means $\pm$ S.E.M. for 3 separate experiments each with a different membrane preparation: *significant difference ($P < 0.01$) between +/+ and ob/ob samples for the same tissue. AC basal activity was measured as described in the Experimental section; for measurement of maximal activity, 10 mM-Mn²⁺ and 0.1 mM-forskolin were added to the mixture.

Tissue	Animal	Na ⁺ ,K ⁺ -ATPase		5'-Nucleotidase		AC (pmol/min per mg)	
		K_m (μ M)	V_{max} (μ mol/ μ g)	K_m (μ M)	V_{max} (nmol/ μ g)	Basal	Maximal
Liver	+/+	0.8 $\pm$ 0.04	23.9 $\pm$ 3.02	29.5 $\pm$ 2.4	3.37 $\pm$ 0.65	4.2 $\pm$ 0.7	753 $\pm$ 146
	ob/ob	0.8 $\pm$ 0.05	22.0 $\pm$ 3.45	28.8 $\pm$ 3.1	3.15 $\pm$ 0.74	4.4 $\pm$ 1.1	832 $\pm$ 124
Brain	+/+	1.1 $\pm$ 0.05	26.2 $\pm$ 2.6	20.1 $\pm$ 1.0	0.82 $\pm$ 0.11	(0.9 $\pm$ 0.1) $\times 10^3$	(2.1 $\pm$ 0.5) $\times 10^3$
	ob/ob	1.1 $\pm$ 0.05	33.3 $\pm$ 1.6*	19.7 $\pm$ 0.2	0.84 $\pm$ 0.11	(0.9 $\pm$ 0.1) $\times 10^3$	(2.9 $\pm$ 0.4) $\times 10^3$

Table 2. Effect of cholera toxin on the incorporation of ADP-ribose in liver and brain membranes of lean (+/+) and obese (ob/ob) mice

Membranes were incubated with cholera toxin and [³²P]NAD⁺ as described in the Experimental section. After protein separation by electrophoresis, the gels were autoradiographed. The data are expressed in fmol/mg of protein, corrected for the time of exposure, as described in the Experimental section. Each sample was assayed at three different protein concentrations. The data are means $\pm$ S.E.M. for 5 separate determinations with different membrane preparations: 42 kDa and 46 kDa refer to the substrates with those molecular masses respectively; 'Total' is the sum of the two. *Significant difference between +/+ and mutant for the same tissue.

Tissue	Animal	Incorporation (fmol/mg of protein)			Ratio 42 kDa/ 46 kDa
		42 kDa	46 kDa	Total	
Liver	+/+	14.3 $\pm$ 0.6	14.4 $\pm$ 0.9	28.6 $\pm$ 1.1	1.0 $\pm$ 0.06
	ob/ob	7.1 $\pm$ 0.8*	6.2 $\pm$ 0.9*	13.3 $\pm$ 1.5*	0.9 $\pm$ 0.07
Brain	+/+	6.0 $\pm$ 1.7	15.3 $\pm$ 4.7	21.5 $\pm$ 5.4	0.41 $\pm$ 0.04
	ob/ob	8.2 $\pm$ 1.8	27.5 $\pm$ 8.2	35.6 $\pm$ 6.0	0.30 $\pm$ 0.03

Table 3. ADP-ribosylation of pertussis-toxin substrates in membranes

Liver and brain membranes from lean (+/+) and obese (ob/ob) mice were ADP-ribosylated with activated pertussis toxin as described in the Experimental section. After protein separation by electrophoresis, the gels were autoradiographed. The data are expressed in fmol/mg of protein and corrected for the time of exposure, as described in the Experimental section. Each sample was assayed at three different concentrations of protein. The data are means $\pm$ S.E.M. for 6 experiments each with different membrane preparations: *significant difference between lean and obese samples.

Animal	Incorporation (fmol/mg of protein)	
	Liver	Brain
+/+	289 $\pm$ 80	1058 $\pm$ 252
ob/ob	165 $\pm$ 45*	834 $\pm$ 275

To determine whether the changes observed in the liver membranes of the ob/ob mouse were attributable to lower protein levels or to lower activity, immunodetection with specific antibodies was used to quantify the various isoforms.

Fig. 1. ADP-ribosylation of G α subunits in liver (a, b) and brain (c, d) membranes

Representative autoradiographs of the data shown in Tables 2 and 3. (a) and (c) represent cholera-toxin substrates, and (b) and (d) pertussis-toxin substrates. In each case, the three lanes represent different concentrations of proteins from +/+ tissues, and the last three lanes represent different concentrations of protein from ob/ob tissue. The markings at the left of each radiograph indicate the placement of the molecular-mass markers, 48 kDa (upper) and 30 kDa (lower).

Identification of the cholera- and pertussis-toxin substrates species by immunodetection

Quantification of the cholera-toxin substrates was also done

Table 4. Immunodetection of cholera-toxin substrates in liver and brain membranes of lean and obese mice

After electrophoresis, the protein bands were transferred to nitrocellulose membranes and subjected to specific antibodies, as described in the Experimental section. After reaction with the ^{125}I -labelled γ -globulin, the nitrocellulose membranes were radioautographed. The results are expressed as arbitrary units, corrected for the amount of protein in the sample and for the time of exposure. Each sample was assayed at three different protein concentrations and the data given are means $\pm$ S.E.M. of at least four different experiments: *significant difference between $+/+$ and ob/ob samples for a given tissue.

Tissue	Animal	Densitometry (arbitrary units/mg of protein)			Ratio 42 kDa/46 kDa
		42 kDa	46 kDa	Total	
Liver	$+/+$	25.0 ± 3.5	25.5 ± 4.2	45.1 ± 8.0	0.94 ± 0.06
	ob/ob	$14.0 \pm 1.2^*$	$11.5 \pm 2.0^*$	$24.0 \pm 6.9^*$	1.08 ± 0.09
Brain	$+/+$	21.8 ± 2.5	38.7 ± 4.2	59.8 ± 7.6	0.61 ± 0.08
	ob/ob	20.0 ± 2.3	45.0 ± 8.8	67.4 ± 8.4	0.48 ± 0.06

Table 5. Immunodetection of pertussis-toxin substrates in liver and brain membranes of lean and obese mice

Immunodetection was carried out as described in the legend to Table 4 and in the Experimental section. After reacting with the ^{125}I -labelled γ -globulin, the nitrocellulose membranes were radioautographed. The results are expressed as arbitrary units corrected for the amount of protein in the sample and for the time of exposure, as described in the Experimental section. Each sample was assayed at three different protein concentrations. The following antibodies were used: for $G_{i\alpha 1}$ and $G_{i\alpha 2}$, NEI-801; for $G_{i\alpha 3}$ and G_o , NEI-803; and for G_o , NEI-804. Each experiment was repeated at least three times: *significant difference between lean and obese groups. + = the peptide was present, but could not be quantified because of interference by other isoforms; nd = not detectable within the limits of our experiments.

Tissue	Animal	Densitometry (arbitrary units/mg of protein)			
		$G_{i\alpha 1}$	$G_{i\alpha 2}$	$G_{i\alpha 3}$	G_o
Liver	$+/+$	nd	19.0 ± 2.8	+	nd
	ob/ob	nd	$12.6 \pm 3.0^*$	+	nd
Brain	$+/+$	64.1 ± 10.0	35.2 ± 9.5	+	92.5 ± 19.8
	ob/ob	62.5 ± 15.0	37.1 ± 12.8	+	80.0 ± 20.5

by immunodetection using an antibody to the C-terminus of the $G_s\alpha$ peptides (NEI-805; see Simonds *et al.*, 1989). As expected, two isoforms of $G_s\alpha$ were detected in both liver and brain membranes. In both tissues, these findings confirm the results obtained by ADP-ribosylation: the abundance of both $G_s\alpha$ isoforms was significantly lower in the liver membranes of obese than of lean mice, whereas there was no difference in brain membranes (Table 4, Fig. 2).

Three different antibodies were used to determine the identity of the pertussis-toxin substrates. NEI-801 was used to detect $G_{i\alpha 1}$ and $G_{i\alpha 2}$; it has approximately equal affinity for both and little cross-reactivity with $G_{i\alpha 3}$. NEI-803 is more reactive towards $G_{i\alpha 3}$ and G_o , but it has significant cross-reactivity with $G_{i\alpha 2}$ and $G_{i\alpha 1}$ also. NEI-804 detects only G_o (see Simonds *et al.*, 1989). These experiments showed that in the liver membranes (Table 5, Fig. 2) only one peptide, identified as $G_{i\alpha 2}$, was detectable in measurable amounts, as shown by its reactivity with NEI-801 and its relative mobility, compared with brain membranes. $G_{i\alpha 2}$

Fig. 2. Immunodetection of isoforms of α -subunits in liver (a-c) and brain (d-f) membranes

Representative autoradiographs of the data shown in Tables 4 and 5. (a) and (d) represent cholera-toxin substrates detected with NEI-805. (b) and (e) represent $G_{i\alpha 1}$ and $G_{i\alpha 2}$, detected with NEI-801 after electrophoresis on high-resolution gels. (c) and (f) represent G_o , detected with NEI-804. In each case, lanes 1-3 are from $+/+$ tissues and lanes 4-6 from ob/ob tissues. Where applicable, lane 7 represents a membrane sample from brain, used as a positive control. The markings at the left of each radiograph indicate the placement of the molecular-mass markers, 48 kDa (upper) and 30 kDa (lower).

was found at significantly lower levels in the liver membranes of obese than of lean mice (Table 5). NEI-804 detected no peptide in the liver, but detected the peptide of appropriate size in a sample of brain membrane used as control, indicating that, if present in liver membranes, G_o is below the limit of detection of our assay. Although $G_{i\alpha 3}$ was detected with NEI-803, the levels were relatively low, and the cross-reactivity with other isoforms hampered its quantification.

In brain membranes, four peptides were identified, of which G_o and $G_{i\alpha 1}$ appeared to be the most prominent, followed by $G_{i\alpha 2}$. $G_{i\alpha 3}$ seemed to be present at low levels, but, again, its quantification was hampered by significant cross-reactivity with the other isoforms. There were no significant differences between lean- and obese-mouse membranes.

α -subunit mRNA levels

Probing RNA extracts from brain with the rat and mouse α -subunit cDNAs revealed that mRNAs were expressed for each of

Table 6. Expression of G α subunits in liver and brain

Total RNA preparations from brain and liver were probed with rat and mouse cDNAs as described in the Experimental section. The probes are full-length cDNAs, except for rat α_s , which generated two *Eco*RI fragments. After hybridization and autoradiography the mRNAs were compared with mouse heart size markers. tr means that trace amounts were found consistently. The same mRNAs were expressed in tissues from lean and obese mice.

cDNA probe		Size of probe (kb)	mRNA expressed (kb)
Mouse	G α_2	1.3	2.3
	G α_s	1.1	1.9, tr 4.0
Rat	G α_1	1.9	3.5
	G α_2	1.8	2.3
	G α_3	3.0	3.5
	G α_o	2.1	4.0
	G α_s	1.2, 0.6	1.9, tr 4.0

the cDNA probes used. Table 6 shows the species of mRNA expressed for each subunit. The faint band corresponding to a 4.0 kb mRNA for G α_s was present consistently when either mouse or rat G α_s cDNA was used as probe, and was expressed in other tissues such as kidney and testis, but not in the HGFu and Ob17 preadipocyte cell lines (N. McFarlane-Anderson & N. Bégin-Heick, unpublished work); its significance is not known at present. In liver, G α_s , G α_2 and G α_3 are predominant, and G α_1 is present, but G α_o is barely detectable. This pattern is consistent with that observed with the immunodetection experiments. In brain, the levels of G α_s , G α_2 and G α_o appeared to be higher than those of G α_1 and G α_3 . All the subunits were more abundant in brain than in liver (Figs. 3a and 3b). Although the observed levels of the individual subunits could be a function of the different affinities of the various cDNA probes for each mRNA species, the fact that similar data were obtained by immunodetection and cDNA probing indicates that they fairly represent the actual distribution of G α subunits within a given tissue.

Since the same cDNAs were used to probe lean- and obese-mouse tissue, it is clear that there is no difference in the levels of mRNAs encoding the α -subunits in tissues of lean or obese mice, although the levels of some immunodetectable peptides and the ADP-ribosylation activity are lower in the liver of the obese than of the lean mouse.

β -subunit cDNA

The sequence of the 369 bp fragment corresponded to nucleotides 178–546 of the translated region of the human liver cDNA (amino acids 60–182) and it shows 94 % similarity to the human nucleotide sequence and the deduced amino acid sequence and 100 % identity with the liver sequence (Fig. 4).

β -subunit mRNA levels

Northern-blot analysis revealed the presence of two mRNA species, 2.9 and 1.8 kb in length, which hybridized with the mouse probe (Fig. 5). The level of expression of the β_1 and β_2 species varied between the brain and liver, but the proportion of 2.9 kb to 1.8 kb species was approximately the same. These mRNAs are similar in size to those described for human liver and rat adipose tissue (Fong *et al.*, 1987; Ros *et al.*, 1989a). There were no differences in the levels of mRNA encoding the β -subunit in tissue from lean and obese animals, as confirmed by dot-blot analysis (Fig. 6). This is consistent with our finding that there was no difference in the level of immunodetectable β -peptide in the adipocytes (Bégin-Heick, 1990) or in testicular

Fig. 3. Identification of the mRNA species corresponding to the α -subunits

(a) Northern-blot analysis of G α_2 and G α_s mRNA. Samples (10 μ g) of total RNA from brain (lanes 1 and 2) and liver (lanes 3 and 4) was subjected to electrophoresis as described in the Experimental section. After transfer to nylon membranes, hybridization was carried out using either the (a) mouse G α_2 or (b) rat G α_s probes. Size markers are indicated in kb. In each case, lanes 1 and 3 are from tissues of lean mice, and lanes 2 and 4 from tissues of obese mice. The data shown are representative of 3 similar experiments. (b) Dot-blot analysis of G α_1 , G α_3 and G α_o mRNA. Samples (0.25, 0.5, 1.0 and 2.0 μ g respectively) of total RNA from brain (lanes 1–4) or liver (lanes 5–8) were denatured, applied to nylon membranes and hybridized with cDNA probes: (1) G α_1 ; (2) G α_3 ; (3) G α_o . Column A, +/+; column B, *ob/ob*. *Brain RNA concentrations of 0.125, 0.25, 0.5 and 1.0 μ g respectively.

membranes (McFarlane-Anderson & Bégin-Heick, 1991) from these animals.

DISCUSSION

The ratio of G-protein subunits is known to vary with the tissue and with the state of differentiation of the cell (Watkins *et al.*, 1987; Milligan, 1989) and to be altered through the influence of various hormones (Malbon *et al.*, 1988). Insulin status has been proposed as a factor in the control of the profile of G-proteins in tissues, in particular adipocytes and liver, although

Fig. 4. Composite nucleotide sequence of the mouse cDNA encoding β -subunit and its deduced amino acid sequence

Nucleotides are numbered in the 5' to 3' direction. The nucleotide sequences of the human and bovine adrenal $\beta 2$ subunit and its deduced amino acid sequence are presented for comparison.

Hormones such as thyroid and glucocorticoid are known to alter cellular responses to other hormones such as catecholamines and insulin. These 'permissive' hormones exert an influence on the character of the G-proteins expressed in target cells. Thus both thyroid-hormone status and glucocorticoid status were found to affect the levels of α - and β -subunits of G_s and G_i as well as that of the β -adrenergic receptor subtypes and the catalytic unit of AC in many cells (see Malbon *et al.*, 1988). As the status of insulin, glucocorticoids and thyroid hormones is profoundly altered in the *ob/ob* mouse, it was natural to ask how this might affect G-protein patterns in tissues in relation to AC modulation.

and adipocyte enzyme are different, they suggest a defect in inhibitory regulation in both of these tissues (Bégin-Heick, 1985; Bégin-Heick & Welsh, 1988). For the adipocyte, the quantification of G-proteins supported the kinetic data (Bégin-Heick, 1990).

The results presented above show that the levels of $G_s\alpha$ and $G_i\alpha$ peptides, as measured in terms of ADP-ribosylated and immunologically reactive peptides, are both lower in the liver membranes of the *ob/ob* mouse. Although this is similar to the pattern found in adipocyte membranes, it is surprising in view of the greater responsiveness of AC to the stimulatory action of catecholamines in liver membranes of obese than of lean mice and of their similar response to activation by glucagon (Bégin-Heick & Welsh, 1988). The aggregated data therefore suggest that even a 50 % lower level of $G_s\alpha$ subunits does not prevent the activation of AC, although it may alter the kinetics of its response to ligands. As both the short and the long forms of $G_s\alpha$

Fig. 5. Identification of the mRNA species corresponding to the β -subunits

Samples of total RNA from brain (5 μ g; lanes 1 and 2) and liver (10 μ g; lanes 3 and 4) were subjected to electrophoresis as described in the Experimental section. After transfer to nylon membranes, hybridization was carried out using the β -subunit 369 bp cDNA probe. Size markers are indicated in kb. Lanes 1 and 3 are samples from lean mice and lanes 2 and 4 samples from obese mice. The data shown are representative of 4 similar experiments.

Fig. 6. Dot-blot analysis of the β -subunit RNA

RNA from lean- and obese-mouse brain (5 μ g) and liver (10 μ g) was denatured and analysed as described in the Experimental section. Autoradiograms were exposed for 16–24 h and the intensity of the radioactive spots was quantified by densitometry. The autoradiogram shown is representative of 4 similar ones. For brain (a), 0.25, 0.5, 1.0, 1.25 and 2.5 μ g of RNA and for liver (b) 0.5, 1.0, 1.25, 2.5 and 5.0 μ g of RNA were analysed in duplicate. Group 1 represents samples from +/+ and group 2 samples from ob/ob tissue. In arbitrary units, the means $\pm$ S.E.M. of the 4 experiments were: brain +/+ = 69.0 ± 6.0 , ob/ob = 72.0 ± 9.3 ; liver, +/+ = 62.0 ± 12.4 ; ob/ob = 65.5 ± 10.9 .

are decreased, the explanation cannot be found in the different properties of these two forms (see Freissmuth *et al.*, 1989). It is therefore difficult to ascribe functional significance to the loss of $G_s\alpha$ peptide in the obese-mouse liver membranes at present. As shown by others (Chang & Bourne, 1989), high levels of AC

activation can be attained with decreased levels of $G_s\alpha$. The enhancement of AC activity by β -adrenergic agonists in the obese tissue, in spite of a 50% decrease in $G_s\alpha$, suggests that the type and/or the abundance of β -adrenergic receptors may be different in the obese- and lean-mouse liver membranes, and may be more important than G_s levels in determining the level of stimulation of cyclase. This possibility requires investigation.

The decreased levels of $G_s\alpha$ are consistent with the loss of inhibition of AC activity in the obese-mouse liver membranes (Bégin-Heick & Welsh, 1988). In other models of obesity and insulin resistance, a decrease in ADP-ribosylation of G_{i2} was linked to loss of inhibition of AC and ascribed to increased phosphorylation of the peptide *in vivo* (Pyne *et al.*, 1989; Houslay *et al.*, 1989; Bushfield *et al.*, 1990a,b). In the present case, the decrease in ADP-ribosylation is paralleled by a decrease in the immunodetectable peptides. This was not the case in the systems mentioned above, suggesting that the defect in $G_s\alpha$ observed in the liver of ob/ob mice is not caused by such a modification.

The state of association of the heterotrimeric form of G-proteins is important in determining the ability of α -subunits to be ADP-ribosylated (see Gilman, 1987; Casey *et al.*, 1989). There is, however, no reason *a priori* to expect that alterations in the ability to form the heterotrimer would affect the levels of peptides assessed by immunodetection, and therefore factors that might influence the formation of the heterotrimeric form (e.g. an overabundance of $\beta\gamma$ subunits) cannot be offered as an explanation for our findings.

Much of the published data on G-protein patterns in tissues is based on measurements of ADP-ribosylation and immunodetection of peptides (Watkins *et al.*, 1987; Ros *et al.*, 1988, 1989a,b; Ohisalo & Milligan, 1989; Garcia-Sainz *et al.*, 1989; Bégin-Heick, 1990), but some studies have been conducted at the mRNA level (Watkins *et al.*, 1989; Rapijko *et al.*, 1989; Ros *et al.*, 1989a,b). The finding that the level of peptide (immunodetection) as well as the activity (ADP-ribosylation) was altered in liver membranes of the ob/ob mouse led us to investigate the level of expression of mRNA corresponding to these peptides. In the studies described here, we show that the mRNAs expressed in a given tissue correspond to specific immunodetectable peptides, but that the level of the mRNA and the level of the peptide are not necessarily congruent. Like ourselves, others have obtained disparate results between the data obtained at the protein and mRNA levels (Watkins *et al.*, 1989; Silbert *et al.*, 1990). The differences in the patterns of ADP-ribosylation and immunodetection prevalent in the liver of lean +/+ and ob/ob mice do not seem to be attributable to different levels of the mRNA coding for these peptides. We therefore conclude that the transcription process is normal.

Although transcription appears to occur normally, the possibility remains that the translation process, the half-life of the peptides, their release from the membrane or a post-translational modification is altered in response to hormonal or other influences. Cross-regulation of the steady-state level of G-protein subunits has been reported in various cells (Chang & Bourne, 1989; Ransnas *et al.*, 1989; Hadcock *et al.*, 1990; Levine *et al.*, 1990; Silbert *et al.*, 1990; Haraguchi & Rodbell, 1990) and may be under hormonal influence.

The lack of correlation between levels of mRNA and the quantity of G-proteins present in the cell was also noted by Silbert *et al.* (1990) and could reflect the fact that quantification is carried out on membrane preparations, although these proteins may not be exclusively membrane-bound (see Milligan, 1989). In this respect, it would be interesting to assess whether there is a difference between lean- and obese-mouse tissues in the release of these peptides in the cytosolic fraction of liver or adipocytes.

In view of the similarities observed between the adipocyte and liver membranes in terms of ADP-ribosylated and immunodetectable peptides, it was surprising to find that, in brain, the abundance of these peptides was not significantly different in lean and *ob/ob* mice. The data reported here are from whole brain membrane preparations. It is possible that differences could be found in discrete brain regions. Immunocytochemical studies could be helpful in this respect.

Although there are some similarities between the findings in *ob/ob* mice (Bégin-Heick, 1990; the present work) and *fa/fa* rats (Houslay *et al.*, 1989; Bushfield *et al.*, 1990a,b), in terms of decreased G-protein levels in the mutant, the full significance of these changes in G-protein patterns as they relate to AC regulation or other cellular functions still has not been elucidated. The stoichiometry of the various components of the AC cascade is not yet established, and it remains to be determined whether different G-protein isoforms play distinct roles in the cell.

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