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Differentiation of cultured brown adipocytes is associated with a selective increase in the short variant of $G_s\alpha$ protein. Evidence for higher functional activity of $G_s\alpha S$

Lenka Bouřová [a,b,c], Zuzana Pešanová [a,b,c], Jiří Novotný [a,b,c], Tore Bengtsson [b,c], Petr Svoboda [a,b,c,*]

[a] *Institute of Physiology, Czech Academy of Sciences, Videáňská 1083, 142 20 Prague 4, Czech Republic*
[b] *Department of Physiology, Faculty of Natural Sciences, Charles University Viničná 7, 128 00 Prague 2, Czech Republic*
[c] *The Wenner-Gren Institute, The Arrhenius Laboratories F3, Stockholm University, S-106 91 Stockholm, Sweden*

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Abstract

In order to examine whether the differentiation process in brown adipocytes cultivated in primary culture is associated with substantial alterations in the complement of G proteins, the levels of these proteins were investigated with immuno-electrophoretic techniques in membrane preparations from proliferating and differentiated cultured mouse brown adipocytes. We observed that differentiation was associated with a dramatic (more than threefold) increase in the short variant of $G_s\alpha$ protein ($G_s\alpha S$). The long variant of $G_s\alpha$ ($G_s\alpha L$), as well as $G_i1\alpha$, $G_i2\alpha$, $G_q\alpha$, $G_{11}\alpha$ and Gβ subunit proteins remained unchanged whereas $G_i3\alpha$ protein was decreased. These changes were accompanied by marked increase in isoprenaline-, forskolin- as well as manganese-stimulated adenylyl cyclase. Thus, the marked increase in β-adrenergic responsiveness of fully differentiated confluent brown adipocytes (day 8–9), as compared with that of proliferating undifferentiated cells of 'fibroblast phenotype' (day 3–4), is associated with a significant increase in the relative proportion between the short and long variants of $G_s\alpha$ (the $G_s\alpha S/G_s\alpha L$ ratio) along with a decrease in $G_i3\alpha$ protein. These data also suggest that the short variant of $G_s\alpha$ exhibits higher functional activity than the long variant of this G protein. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Differentiation; Brown adipocytes; Culture; G proteins; $G_s\alpha$; $G_i\alpha$; Isoprenaline; Adenylyl cyclase

1. Introduction

The recruitment process of brown adipose tissue occurs around birth in all mammalian species (Nedergaard et al., 1986, 1992). It also occurs under physiological conditions such as cold acclimation (Himms-Hagen, 1986) and under the influence of certain diets (Rothwell and Stock, 1986). It consists of both cell proliferation and differentiation (McIntyre et al., 1996). It is generally accepted that these processes are initiated by β-adrenergic signals and noradrenaline appears to be the major regulator of proliferation, differentiation as well as thermogenesis of BAT cells (Nedergaard et al., 1992, 1995; Cannon and Nedergaard, 1996).

Hormonal messages received by adrenergic receptors are transmitted through the relevant G proteins, which serve as molecular switches for communicating extracellular information into the target cell (Taylor, 1990; Neer, 1995). The modulatory roles of the G proteins in different cellular processes have also been clearly estab-

Abbreviations: BAT, brown adipose tissue; DTT, dithiothreitol; EGF, epidermal growth factor; G protein, heterotrimeric guanine nucleotide-binding regulatory protein; the $G_s\alpha$, α subunit of the stimulatory G protein; $G_s\alpha L$ and $G_s\alpha S$, long and short variant of $G_s\alpha$, respectively; $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$, α subunits of pertussis toxin (PT) sensitive inhibitory G proteins; $G_q\alpha$ and $G_{11}\alpha$, α subunits of PT insensitive G proteins G_q and G_{11}, respectively; GPCR, G protein-coupled receptor; PBS, phosphate buffered saline; PM, plasma membrane; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, polyacrylamide gel electrophoresis in sodium laurylsulfate; TBS, Tris buffered saline; T3, triiodothyronine.

* Corresponding author. Tel.: +420-2-4752478/2533; fax: +420-2-4752488.

E-mail address: svobodap@biomed.cas.cz (P. Svoboda).

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lished (Helms, 1995; Wang and Malbon, 1996; Malbon, 1997). It is therefore of interest to investigate the behaviour of these proteins during differentiation and proliferation processes. Marked alterations in the levels of the stimulatory (G_s) as well as inhibitory (G_i) G proteins have been described during, e.g. adipogenetic differentiation of mouse embryo 3T3 L1 cells (Watkins et al., 1987, 1989; Su et al., 1993).

In this study we have examined the changes in G protein levels during the transition switch of in vitro cultivated mouse brown fat precursor cells into mature fully differentiated adipocytes. The data indicated a substantial increase in the short variant of $G_s\alpha$ protein $G_s\alpha S$. This increase was rather selective as it was associated with little or no change of other G proteins-the long variant of $G_s\alpha$ ($G_s\alpha L$), as well as $G_i1\alpha$, $G_i2\alpha$, $G_q\alpha$, $G_{11}\alpha$ and $G\beta$ proteins remained unchanged. The only exception was represented by $G_i3\alpha$ which was decreased. In the next step of our experimentation, these structural changes were correlated with G protein functional activity, which was determined as isoprenaline- and forskolin-stimulated adenylyl cyclase. It is well known that forskolin-stimulated adenylyl cyclase activity depends strongly on the presence of G proteins which has been clearly documented by the existence of high-affinity forskolin binding sites in a number of mammalian tissues cells (Stengel et al., 1982; Darfler et al., 1982; Barber, 1988; Alousi et al., 1991). As the direct measure of functional activity of catalytic unit of adenylyl cyclase, the manganese-stimulated enzyme was also determined. In this way, the full structure-function correlation studies of G protein complement in undifferentiated and fully-differentiated adipocytes was performed.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), HEPES, l-glutamine and antibiotics were from ICN Biomedicals, new-born calf serum and insulin were purchased from LabFab and NovoNordisk. All other chemicals were from Sigma and they were of the highest quality available.

2.2. Cell isolation and culture

Brown fat precursor cells were isolated from young mice and cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% new-born calf serum, 50 IU/ml penicilin, 50 μg/ml streptomycin, 25 μg/ml sodium ascorbate and 4 nM insulin as described before (Rehnmark et al., 1990; Bronnikov et al., 1992). Primary cultures of proliferating (day 3–4) and confluent differentiated (day 8–9) cells were washed once in PBS, harvested with a cell scraper into 20 mM Tris (pH 7.4), 1 mM EDTA containing 1 mM PMSF and frozen at −80°C.

2.3. Preparation of crude membranes

After thawing, the cells were homogenised on ice in a tightly-fitting Potter-Elvehjem (Teflon-glass) homogeniser for 5 min at 1800 r.p.m. and the homogenate was centrifuged for 1 h at 75 000 r.p.m. ($250\,000 \times g$) in a Beckman T100 centrifuge, 2–4°C. The sediment (crude membranes) was resuspended in 20 mM Tris (pH 7.4), 1 mM EDTA containing 1 mM PMSF and stored at −80°C until use.

Low-speed supernatant (1500 r.p.m., 5 min) prepared from rat brain cortex or guinea-pig heart homogenates (20% w/v) was centrifuged for 30 min at 40 000 r.p.m. in a Beckman L5 centrifuge and the resulting sediment was resuspended in 20 mM Tris (pH 7.4), 1 mM EDTA, 1 mM PMSF. These membranes served as positive standards for G_i/G_o (brain) and G_s (heart) class of G proteins when using an immunoblot analysis of samples of brown adipocytes.

2.4. SDS-polyacrylamide gel electrophoresis

Crude membranes prepared from proliferating preconfluent cells (day 3–4) and confluent, differentiated cells (day 8–9) were solubilised in Laemmli buffer [50 mM Tris (pH 8.0), 0.5% SDS, 0.5% DTT, 30% urea, 0.005% bromophenol blue] and loaded (50 μg per lane) on standard [10% (w/v) acrylamide/0.26% w/v bis-acrylamide] or high-resolution, urea-polyacrylamide [12.5% (w/v) acrylamide/0.0625% (w/v) bis-acrylamide containing 6 M urea] gels where indicated. SDS-PAGE was carried out overnight at 60 V (standard gel) or 100 V (urea gel) as described earlier in detail (Svoboda and Milligan, 1994). Molecular mass determinations were based on prestained molecular mass markers (Sigma, SDS 7B).

2.5. Production of antisera and immunoblotting

Antisera used in this study are rabbit polyclonal antipeptide sera which were raised in rabbits obtained from VELAZ, Prague (CS, SG, CQ, BN) or in New Zealand White rabbits, Glasgow (I3B; a generous gift of Prof. G. Milligan, Glasgow University, Glasgow, UK). Immunization was performed with a glutaraldehyde conjugate of keyhole limpet hemocyanin and synthetic peptides specific for a given type of G protein. Antiserum CS was raised against a peptide corresponding to the C-terminal decapeptide of $G_s\alpha$, RMHLRQYELL ($G_s\alpha$, amino acids 385–394) as described before (Mullaney et al., 1993a; Bouřová et al.,

1999a). Antiserum SG was raised against a synthetic peptide corresponding to the C-terminal decapeptide of transducin, KENLKDCGLF ($G_t\alpha$, amino acids 341–350) which identifies $G_i1\alpha$ and $G_i2\alpha$ proteins in the brain as well as variety of other mammalian tissues (Goldsmith et al., 1987; Mitchell et al., 1989) including brown adipose tissue (Svoboda et al., 1996). Antiserum CQ (Mitchell et al., 1989) was produced against peptide QLNLKEYNLV representing the C-terminal decapeptide which is conserved between $G_q\alpha$ and $G_{11}\alpha$ (Strathmann and Simon, 1990). This antiserum identifies both polypeptides equally in transfected cell lines (Mullaney et al., 1993a,b) as well as in brown adipose tissue (Bouřová et al., 1999b). Antiserum I3B was raised against a peptide corresponding to the C-terminal region of $G_i3\alpha$, KNNLKECGLY ($G_i3\alpha$, amino acids 345–354) (Mitchell et al., 1989). This antiserum was used before for detection of $G_i3\alpha$ in isolated plasma membranes from hamster brown adipose tissue (Bouřová et al., 1999b). Antiserum BN was raised against a synthetic polypeptide corresponding to the N-terminal decapeptide of the $G\alpha1$ subunit according to Green and Johnson (1989) and was tested previously for detection of $G\alpha$ subunits in hamster brown adipose tissue (Svoboda et al., 1996).

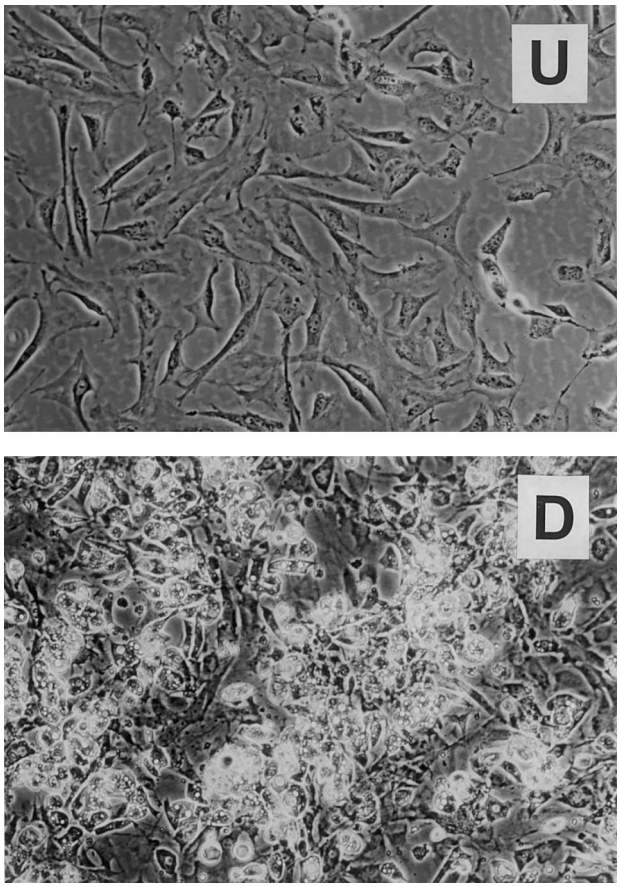


Fig. 1. Representative micrographs of proliferating brown preadipocytes (day 3–4) and fully differentiated brown adipocytes (day 8–9). Precursor cells isolated from the brown adipose tissue of 3-week old mice were seeded in culture flasks and grown until complete maturation. (U) undifferentiated, proliferating preadipocytes after 3–4 days in culture, (D) fully differentiated cells after 8–9 days in culture.

After SDS-PAGE, the resolved proteins were transferred to nitrocellulose membranes (Schleicher and Schuell), blocked with 4% bovine serum albumin for 1 h and then incubated with the relevant G protein-specific primary antisera for 2 h. After three 10-min washes in TBS containing 0.03% v/v Tween 20, the secondary antibody, goat anti-rabbit IgG labelled with alkaline phosphatase, was applied for 1 h. After another three 10-min washes in TBS-Tween, the blots were developed in TNM buffer (100 mM Tris-HCl (pH 9.0), 100 mM NaCl and 5 mM $MgCl_2$) containing 5-bromo-4-chloro-3-indolyl phosphate (100 μg/ml) and nitroblue tetrazolium (200 μg/ml) as substrate (Novotný et al., 1995). The immunoblots were scanned (UMAX Astra 610P) and quantitatively analysed by the ImageQuant 3.3 computer program.

2.6. Adenylyl cyclase assay

Adenylyl cyclase activity was determined similarly as described before by Svoboda et al. (1993); the separation of cyclic AMP was performed by alumina column chromatography as described by White (1974). Membrane preparations from differentiated and non-differentiated adipocytes were incubated for 30 min at 30°C in a total volume of 0.1 ml of 70 mM HEPES/HCl (pH 7.4) containing 10 mM $MgCl_2$, 1 mM EDTA, 16 g/ml pyruvate kinase, 10 mM potassium phosphoenolpyruvate, 160 g/ml BSA, 0.1 mM ascorbate, 0.01 mM RO-201724 (phosphodiesterase inhibitor) and 0.15 mM APT plus [^{32}P]ATP (about 1 000 000 c.p.m. per sample). The activating ligands (isoprenaline, forskolin and manganese chloride) were added 5 min before the start of the enzyme reaction by addition of ATP. The reaction was terminated by addition of 0.1 ml of 0.25% SDS/5 mM ATP/ 0.175 mM cyclic AMP plus cyclic [^{3}H]AMP (20 000 c.p.m. per sample as internal standard) and heating for 5 min at 95°C.

2.7. Protein determination

Protein was determined according to Lowry et al. (1951).

3. Results

Primary cultures of cells from mouse brown adipose tissue were grown for up to 9 days in DMEM supplemented with new-born calf serum and insulin (see Section 2), and inspected regularly for morphological

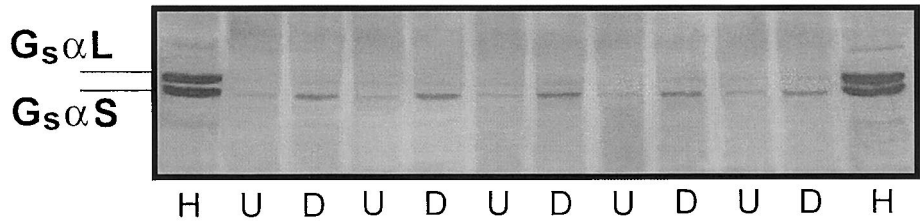


Fig. 2. Identification of $G_s\alpha S$ and $G_s\alpha L$ proteins in brown pre-adipocytes (day 3–4) and mature (day 8–9) brown adipocytes. Five independent (different) membrane preparations isolated from different cell cultures of undifferentiated (U) and differentiated (D) mature brown adipocytes were resolved by standard SDS-PAGE [10% (w/v) acrylamide/0.26% w/v bis-acrylamide] and immuno-blotted with the CS antiserum as described in Section 2. The levels of $G_s\alpha L$ and $G_s\alpha S$ in undifferentiated cells were only slightly above the detection limit on original immunoblots and are hardly discernible on this picture. U, pre-adipocytes (day 3–4), 50 μg per lane; D, differentiated cells after 8–9 days in culture, 50 μg per lane; homogenate of guinea pig myocardium, 20 μg protein.

changes. As seen in Fig. 1, after 3 days in culture, the cells still had relatively large nuclei and lacked the typical morphology of the fat cells. After about 6 days in culture, the cultures became confluent and the cells took on the appearance of mature brown adipocytes, as seen in Fig. 1 (9-day old cells). This spontaneous differentiation process is in accordance with earlier descriptions of this cell culture system (Nechad, 1983; Nechad et al., 1983; Rehnmark et al., 1990). In the present studies, the G protein complement of these two distinct differentiation states of brown fat cells were analysed and contrasted by immunoblot analysis of all the main types of G protein α subunits. Gβ subunits were also determined. This structural analysis was performed in crude membranes (250 000 × g sediment) prepared from cell homogenate of 3-day- and 9-day-old cell cultures as described in Section 2.

3.1. $G_s\alpha S$ and $G_s\alpha L$ proteins

$G_s\alpha$ protein exists in the two different forms (variants) which exhibit slightly different mobility on SDS-polyacrylamide gels-the long ($G_s\alpha L$) and short ($G_s\alpha S$) $G_s\alpha$ proteins (Bray et al., 1986; Graziano et al., 1989; Mattera et al., 1989). The two variants of $G_s\alpha$ were resolved by standard SDS-PAGE using 10% polyacrylamide gels and detected immuno-chemically on blots with a specific antiserum oriented against the C-terminal decapeptide of $G_s\alpha$, CS (Fig. 2). The identification of $G_s\alpha S$ and $G_s\alpha L$ was carried out by comparison of brown adipocytes samples with positive standards prepared from guinea pig myocardial membranes where both variants of $G_s\alpha$ are highly abundant (Roberts et al., 1992; Sethi et al., 1993; Novotny et al., 1999). As shown in Fig. 2 and Table 1, the amount of $G_s\alpha S$ increased in the course of differentiation more than three times as compared to undifferentiated precursor cells. The amount of $G_s\alpha L$ was slightly above detection limit in both types of cells and no significant change could be detected under these conditions.

3.2. $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ proteins

$G_i1\alpha$ and $G_i2\alpha$ proteins were resolved and identified in membrane preparations from the undifferentiated precursor and differentiated mature cells by high-resolution urea-SDS-PAGE (12.5% polyacrylamide gels containing 6 M urea) and immunoblotting with the $G_i1\alpha$ and $G_i2\alpha$-oriented antiserum SG similarly as described before for purified plasma membranes from hamster brown adipose tissue (Mullaney et al., 1993b; Svoboda et al., 1996). Crude membranes prepared from the brain cortex (10 μg per lane) were used as positive standards (Caufield et al., 1994; Svoboda et al., 1996). Membranes prepared from the fully differentiated mature adipocytes exhibited the same amount of $G_i1\alpha$ and

Table 1
G protein levels in undifferentiated pre-adipocytes (day 3–4) and differentiated mature adipocytes (day 8–9)[a]

	Brown pre-adipocytes	Mature brown adipocytes
$G_s\alpha S$	100 ± 18[b]	334 ± 31**
$G_s\alpha L$	74 ± 15[b]	95 ± 11 NS
$G_i1\alpha$	26 ± 1	24 ± 2 NS
$G_i2\alpha$	100 ± 3	92 ± 3 NS
$G_i3\alpha$	100 ± 5	69 ± 6**
$G_q\alpha$	89 ± 5	95 ± 3 NS
$G_{11}\alpha$	100 ± 7	111 ± 5 NS
Gβ	100 ± 13	93 ± 6 NS

[a] The data represent the average of densitometric scannings of five ($G_s\alpha S$ and $G_s\alpha L$), four ($G_i1\alpha$ and $G_i2\alpha$), four ($G_q\alpha$ and $G_{11}\alpha$) and five ($G_i3\alpha$) independent (different) membrane preparations ± SEM resolved on the same blot. The numbers were obtained by densitometric scanning of relevant immunoblot signals. For each antibody, the pre-adipocyte value was set to 100%. If the antibody detected more than one G protein, the level of most abundant was set to 100%. The difference between the two experimental groups was analysed by Student's unpaired *t*-test (** indicates significant change, $P < 0.01$, NS indicates non-significant change).

[b] As the levels of $G_s\alpha L$ and $G_s\alpha S$ in the undifferentiated cells were only slightly above the detection limit, these values have to be considered somewhat uncertain. For the same reason, the value for the relative increase in $G_s\alpha S$ in the mature brown adipocytes may be an underestimate of the real increase.

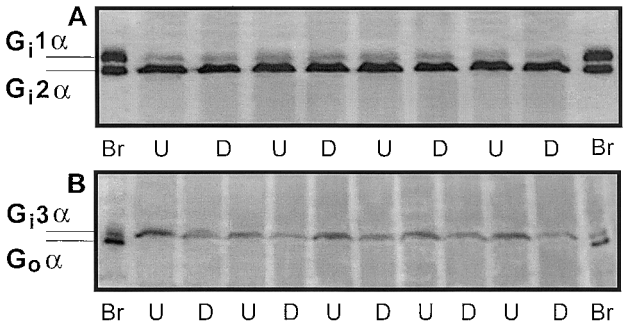


Fig. 3. $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ proteins. Membrane preparations from brown pre-adipocytes (U) and mature brown adipocytes (D) were resolved by high-resolution urea-SDS-PAGE [12.5% (w/v) acrylamide/0.0625% (w/v) bis-acrylamide containing 6 M urea] and immunoblotted with the SG antiserum for detection of $G_i1\alpha$ and $G_i2\alpha$ (A) or I3B antiserum for detection of $G_i3\alpha$ (B). U, pre-adipocytes (day 3–4), 50 μg per lane; D, differentiated cells after 8–9 days in culture, 50 μg per lane; Br, rat brain microsomes, 20 μg protein.

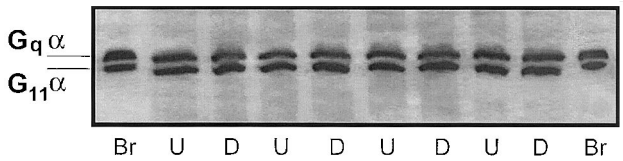


Fig. 4. $G_q\alpha$ and $G_{11}\alpha$ proteins. Membrane preparations from brown pre-adipocytes (U) and mature brown adipocytes (D) were resolved by urea-SDS-PAGE [12.5% (w/v) acrylamide/0.0625% (w/v) bis-acrylamide containing 6 M urea] using the same electrophoretic system as that described for detection of $G_i1\alpha$ and $G_i2\alpha$ proteins and immunoblotted with the CQ antiserum for detection of the $G_q\alpha/G_{11}\alpha$ proteins. U, pre-adipocytes (day 3–4), 50 μg per lane; D, differentiated cells after 8–9 days in culture, 50 μg per lane; Br, rat brain microsomes, 20 μg protein.

$G_i2\alpha$ as the undifferentiated cells (Fig. 3A and Table 1). In contrast, the immuno-chemical signal of $G_i3\alpha$ detected by the I3B antiserum was reduced to 70% after differentiation (Fig. 3B and Table 1). In the brain preparations, I3B antiserum also reacted with a polypeptide exhibiting the higher mobility which is known to represent $G_o\alpha$ protein (Milligan, 1990). The cross-reactivity of I3B antiserum was also noted in our previous study (Bouřová et al., 1999b).

3.3. $G_q\alpha$, $G_{11}\alpha$ and Gβ proteins

The G proteins of the $G_q\alpha/G_{11}\alpha$ family were resolved by the same electrophoretic system (12.5% polyacrylamide gels containing 6 M urea) as that used for the $G_i\alpha/G_o\alpha$ proteins. The antiserum CQ which is oriented against the C-terminal decapeptide of $G_q\alpha/G_{11}\alpha$ and which identifies both proteins equally was used in the immunoblot analysis in the same way as described before when identifying $G_q\alpha/G_{11}\alpha$ proteins in purified plasma membranes from hamster brown adipose tissue (Bouřová et al., 1999b). As in the case of the $G_i1\alpha$ and $G_i2\alpha$ proteins, the membranes prepared from confluent (day 8–9) adipocytes contained the same amounts of the $G_q\alpha/G_{11}\alpha$ proteins as did proliferating cells (day 3–4) (Fig. 4). The same result was obtained for the Gβ subunits, which were identified by BN antiserum (Table 1).

3.4. Isoprenaline-, forskolin- and manganese-stimulated adenylyl cyclase

To obtain the functional correlate to the changes determined by analysis of G protein levels (structural evidence only), isoprenaline-, forskolin- and manganese-stimulated adenylyl cyclase activities were determined in membrane preparations isolated from undifferentiated and differentiated brown adipocytes. The data presented in Fig. 5 clearly show that the mature adipocytes contain significantly higher isoprenaline-stimulated adenylyl cyclase activity than the

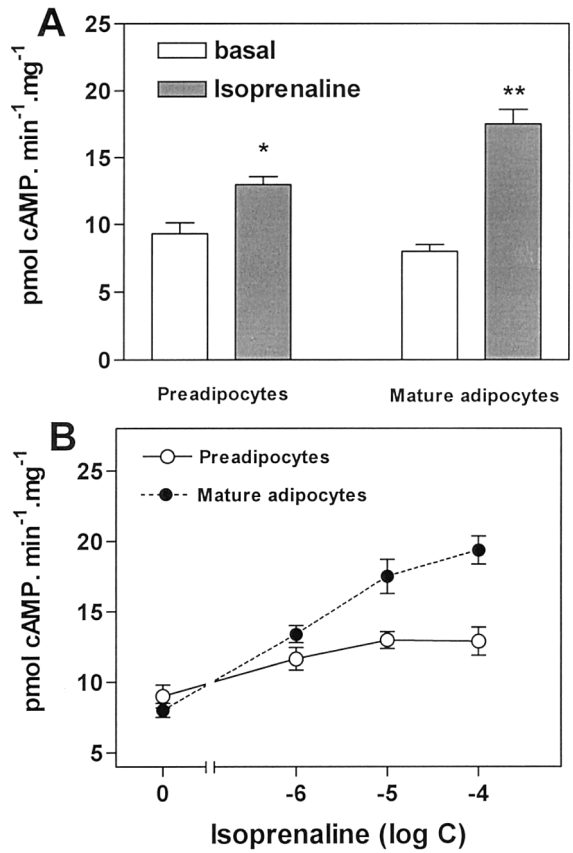


Fig. 5. Isoprenaline-stimulated adenylyl cyclase activity. Isoprenaline (10 μM)-stimulated adenylyl cyclase activity was measured in membrane preparations isolated from undifferentiated and differentiated brown adipocytes as described in Section 2 (A). The dose-response curve of isoprenaline (1–100 μM)-stimulated adenylyl cyclase (B). The results shown are the means of three membrane preparations with assays performed in duplicates (±SEM). The difference between the two preparations was analysed by a Student's *t*-test; ***, **, * indicates significant effect of differentiation ($P < 0.001$, $P < 0.01$ and $P < 0.05$, respectively), NS, non-significant.

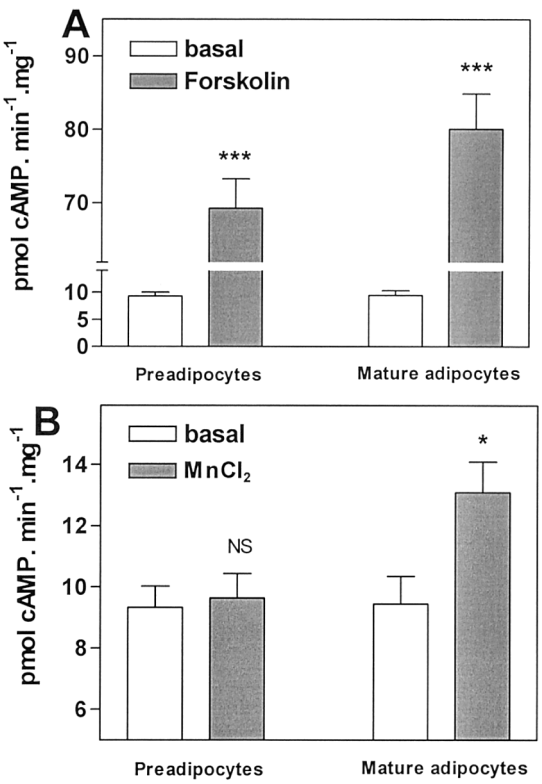


Fig. 6. Forskolin- and manganese-stimulated adenylyl cyclase activity. Forskolin, 10 μM (A) and manganese, 10 mM (B)-stimulated adenylyl cyclase activities were measured in membrane preparations isolated from undifferentiated and differentiated brown adipocytes as described in Section 2. The results shown are the means of three membrane preparations assays performed in duplicates ($\pm$SEM). The difference between the two preparations was analysed by a Student's *t*-test; ***, **, * indicates significant effect of differentiation ($P < 0.001$, $P < 0.01$ and $P < 0.05$, respectively), NS, non-significant.

non-differentiated fibroblasts (day 3–4). As the β-adrenergic stimulation of adenylyl cyclase is mediated via $G_s\alpha$ proteins, this increase may be regarded as an indirect support for an increased activity of $G_s\alpha S$ (when $G_s\alpha L$ is unchanged). The increase of adenylyl cyclase is not confined to β-adrenergic receptor responsive component, but represents more general up-regulation of this enzyme activity. This is evidenced by a parallel increase in forskolin-stimulated adenylyl cyclase (Fig. 6A). It should be mentioned in this respect that forskolin-stimulated adenylyl cyclase activity depends strongly on the presence of $G_s\alpha$ proteins which has been clearly documented by the existence of high-affinity forskolin binding sites in a number of mammalian tissues (Stengel et al., 1982; Darfler et al., 1982; Barber, 1988; Alousi et al., 1991). This type of forskolin binding is not manifested in cells lacking $G_s\alpha$ (cyc^- mutants of S49 lymphoma cells) and is very low in cells containing the functionally deficient $G_s\alpha$ (unc and H21a mutants of S49 lymphoma cells) (Stengel et al., 1982; Darfler et al., 1982; Barber, 1988; Alousi et al., 1991). Finally, as the direct measure of functional activity of catalytical unit of adenylyl cyclase, the manganese-stimulated enzyme was determined (Fig. 6B). This enzyme activity was also increased in differentiated brown adipocytes (day 8–9) when compared with precursor cells (day 3–4). In this way, the full structure-function correlation studies of G protein complement in undifferentiated and fully-differentiated brown adipocytes was performed. The specific increase in the amount of the short variant of the stimulatory G protein (Fig. 2, Table 1), $G_s\alpha S$, was clearly related to an increased functional activity of its effector enzyme, adenylyl cyclase (Figs. 5 and 6).

4. Discussion

4.1. $G_s\alpha$ proteins and adipogenesis

Adipogenesis is the commitment of embryonic stem cells to the highly-differentiated phenotype of the adipocyte, a cell specialised to regulate, in dynamic fashion, lipid storage (Wang and Malbon, 1996). It has been shown that 3T3-L1 fibroblasts undergo differentiation in culture to adipocyte (fat cell)-like phenotype and changes of more than 60 proteins have been found to occur during differentiation (Watkins et al., 1982). The molecular strategies of antisense DNA technology and site-directed mutagenesis revealed that either suppression of $G_s\alpha$ or expression of constitutively activated mutants of $G_i2\alpha$ dramatically accelerate the ability of inducers (such as dexamethasone) to stimulate adipogenesis. The control of this process by the Gs/Gi axis proceeds independently of adenylyl cyclase (Wang and Malbon, 1996).

Analysis of a very similar model, adipogenic differentiation of 3T3-F442 fibroblasts in hormonally defined medium, was found to be associated with a dramatic increase in $G_s\alpha S$ (and relatively small increase in $G_s\alpha L$) (Kilgour and Anderson, 1993). Concomitantly, $G_i1\alpha$ and $G_i2\alpha$ proteins were decreased. These 'structural changes' were associated with a two- to fourfold increase in forskolin-stimulated adenylyl cyclase activity and a 15-fold increase in response to β-adrenergic agonists. The increase in $G_s\alpha S$ (four times) occurring in serum-supplemented medium was further potentiated by the addition of 'inducers' such as growth hormone, insulin, T3 and EGF. Accordingly, Huppertz et al. (1993), comparing 3T3-L1 cells before and after differentiation of the insulin-sensitive phenotype, suggested that the increase in abundance of the short variant of $G_s\alpha$ might be related to a specific function of the adipocyte phenotype. The idea of specific significance of $G_s\alpha S$ for the 'fat cell phenotype' is also supported by results of Denis-Henriot et al. (1996). Mature white adipocytes contain six times more $G_s\alpha S$ than undiffer-

entiated confluent pre-adipocytes while the $G_s\alpha L$ content is the same in both types of cells.

Thus, proliferation and/or differentiation of brown fat cells in serum and insulin supplemented medium, which is characterised by preferential increase in $G_s\alpha S$, decrease in $G_i3\alpha$ and unchanged levels of other trimeric G proteins (including $G_s\alpha L$), appears to be similar to the previously described adipogenetic models of 3T3-F442 or 3T3-L1 fibroblasts. The role of $G_s\alpha S$ and $G_i3\alpha$ proteins in proliferation/differentiation of BAT cells in culture may be related to the ability of GPCRs to induce cellular responses that require transmission of extracellular signals to the nucleus. In general, these responses may include stimulation of DNA synthesis, cellular growth or hypertrophy (Post and Brown, 1996). Also mitogenic responses were found to be mediated by G_i/G_o, G_q/G_{11} or G_s proteins (Meinkoth et al., 1992; LaMorte et al., 1993; Baffy et al., 1994; Crouch and Simson, 1997).

4.2. $G_i\alpha$ proteins and β_3-adrenergic response

As Chaudhry et al. (1994) provided evidence that β_3- but not β_1-adrenergic receptors interact with $G_i\alpha$ proteins in white adipocyte membranes, the increased cAMP responsiveness of differentiated brown adipocytes (β_3) to noradrenaline (Bronnikov et al., 1992) might be related to a decrease in $G_i3\alpha$ described in our present work. It should be mentioned in this respect that adenylyl cyclase inhibition is most commonly mediated via $G_i2\alpha$ protein with a role of $G_i3\alpha$ in some cases (Pairault and Lasnier, 1987; Gerhardt and Neubig, 1991; Houslay, 1991).

The idea that decreased expression of G_i proteins is necessary to elicit maximal agonist stimulation was also supported by Kilgour and Anderson (1993). 3T3-L1 cells maintained at confluence for 12 days, where morphological conversion to adipocytes was negligible (and $G_s\alpha S$ increased no more than four times), exhibited much smaller increase in responsiveness of adenylyl cyclase to isoproterenol as compared to maximally differentiated cells (where $G_s\alpha S$ was increased seven to eight times).

4.3. Functional non-identity of splice variants of $G_s\alpha$

Whilst changes in the levels of G protein expression probably contribute to adenylyl cyclase regulation in brown adipose tissue (Chaudhry and Granneman, 1991, 1992; Svoboda et al., 1993; Chaudhry et al., 1994; Svoboda et al., 1996), the precise contribution of changed levels of particular G proteins remains uncertain. Until recently, there was little evidence to indicate that splice variations in $G_s\alpha$ have more than marginal significance for their properties and function (Graziano et al., 1987, 1989; Mattera et al., 1989; Jones et al., 1990; Freissmuth et al., 1991; O'Donnell et al., 1991). However, abundant literature data (Sternweis et al., 1981; Walseth et al., 1989; Rubenstein et al., 1991; Yagami, 1995) bringing indirect evidence in favour of the idea that $G_s\alpha L$ and $G_s\alpha S$ might not be functionally equivalent (for review see Novotný and Svoboda, 1998), received direct experimental support in work of Seifert et al. (1998). Fusion proteins constructed of β_2-adrenergic receptor (β_2AR) with either $G_s\alpha L$ or $G_s\alpha S$ exhibited different potencies when stimulating both GTPase and adenylyl cyclase activities. Membranes expressing β_2AR-$G_s\alpha S$ had higher basal and (-)-isoprenaline stimulated adenylyl cyclase activity than membranes expressing β_2AR-$G_s\alpha L$ (Seifert et al., 1998).

The data presented in this work clearly support this view and bring further evidence which is obtained from naturally occurring cells and not from artificial transfected cell lines. Therefore, it may be suggested that dramatic increase in $G_s\alpha S$ associated with increased isoprenaline-stimulated adenylyl cyclase activity described in this work is functionally associated with an increased cAMP responsiveness of differentiated brown adipocytes (β_3) to noradrenaline (Darfler et al., 1982).

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