Resolution and identification of $G_q/G_{11}\alpha$ and $G_i\alpha/G_o\alpha$ proteins in brown adipose tissue: effect of cold acclimation

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ABSTRACT

Levels of guanine nucleotide-binding proteins $G_q\alpha$ and $G_{11}\alpha$, which produce receptor regulation of phospholipase C, were measured immunologically in purified plasma membrane fractions of hamster brown adipose tissue (BAT). This was achieved by immunoblotting with antisera (CQ series) that identify these two polypeptides equally, following separation of the plasma membranes using SDS-PAGE in the presence of 6 M urea, i.e. conditions that can resolve $G_q\alpha$ and $G_{11}\alpha$. The ratio of levels of $G_q\alpha$ to $G_{11}\alpha$ was 1:1. A similar approach was used for resolution and identification of $G_o1\alpha$ and $G_o2\alpha$,

the latter representing the prevailing form of $G_o\alpha$ proteins in this tissue. Although clearly recognized in brain microsomes, which were used as positive controls, no detected levels of $G_o*\alpha$ protein were noted. Using specific anti-peptide antibodies directed against the carboxy-terminal decapeptide of $G_i3\alpha$, this G protein was also found to be expressed in BAT tissue. Cold acclimation resulted in reduction of the plasma membrane levels of all these $G\alpha$ proteins.

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INTRODUCTION

Whilst the molecular identities of the G proteins regulating both receptor-mediated stimulation ($G_s\alpha$) and inhibition ($G_i\alpha$) of adenylyl cyclase in brown adipose tissue (BAT) are now firmly established (Granneman *et al.* 1990, Chaudhry & Granneman 1991, 1992, Svoboda *et al.* 1993, 1996a, De Pergola *et al.* 1994), the identification of G proteins responsible for pertussis toxin (PT)-insensitive, receptor-linked phosphoinositidase hydrolysis, $G_q/G_{11}\alpha$ (Mitchell *et al.* 1991, Mullaney *et al.* 1993a,b) is missing. The same applies for the PT-sensitive proteins of the $G_o\alpha$ family (Mullaney & Milligan 1990, Caufield *et al.* 1994) and $G_i3\alpha$ protein of the $G_i\alpha$ family (Mitchell *et al.* 1989, Denis-Henriot *et al.* 1996). Previously, white adipocytes were found to be virtually devoid of $G_o\alpha$ (Hinsch *et al.* 1988, Mitchell *et al.* 1989), while the existence of $G_i3\alpha$ protein has been documented (Mitchell *et al.* 1989).

In this work, using specific anti-peptide antisera generated against synthetic peptides that represent parts of primary sequence of the α -subunits, the $G\alpha$

protein content of hamster BAT was analysed in sucrose density gradient-purified plasma membranes (PM). $G_q\alpha$, $G_{11}\alpha$, $G_o\alpha$ and $G_i3\alpha$ proteins were identified and the membrane density of these G proteins compared in preparations from control and cold-acclimated animals.

MATERIALS AND METHODS

Sucrose (Aristar grade) was from BDH, UK. Phospholipase A_2 was purchased from Sigma, USA (P6139). All other chemicals were of analytical grade purity either from Sigma, Calbiochem, Switzerland or BDH.

Isolation of PM from BAT

PM-enriched fractions were isolated from hamster BAT as described before (Svoboda *et al.* 1993, 1996a, Bouřová *et al.* 1999). Briefly, the tissue homogenate was filtered through a double layer of silk cloth, centrifuged for 5 min at 600 g and applied to a sucrose density gradient consisting of 43, 35,

31, 27, 23 and 19% w/w sucrose (5 ml each). After centrifugation for 1 h at 96 000 *g*, PM fractions were collected from the 23–27% and 27–31% sucrose interphase, diluted 1:1 with 20 mM Tris–HCl, pH 7.4, 1 mM EDTA and sedimented by high-speed centrifugation (1 h at 177 000 *g*). The sediment was rehomogenized at a concentration of 5–12 mg membrane protein per ml and stored at -80°C .

Cell culture and membrane preparations

Clones of human embryonic kidney (HEK) 293 cells (Kim *et al.* 1994, Svoboda *et al.* 1996b) and Chinese hamster ovary (CHO) cells (Mullaney *et al.* 1993a,b, Svoboda & Milligan 1994) were grown and the PM isolated as described before. Rat brain and rat heart microsomes were prepared according to Svoboda & Mosinger (1981).

Primary cultures of BAT were made according to the well-established protocols described by Rehnmark *et al.* (1990) and Bronnikov *et al.* (1992). Harvested cells were washed once in PBS, resuspended in 20 mM Tris, 1 mM EDTA, pH 7.4 (hypotonic lysis TE buffer) and stored at -80°C . The lysed (and frozen once) cell suspension was homogenized in a tightly fitting teflon–glass homogenizer for 5 min at 800 r.p.m., centrifuged for 1 h at 177 000 *g* and membrane sediment suspended in TE buffer.

Production of antisera

Antisera used in this study were rabbit polyclonal anti-peptide antisera which were raised in New Zealand White rabbits in Glasgow (CQ, I3B; a generous gift of Prof. G. Milligan, Glasgow University, Glasgow, UK) or in rabbits obtained from VELAZ, Prague, Czech Republic (IO, CO). Immunization was performed with a glutaraldehyde conjugate of keyhole limpet haemocyanin and synthetic peptides specific for a given type of G protein. Antiserum CQ (Mitchell *et al.* 1989, 1991) was produced against peptide QLNLEKEYNLV that represents the carboxy-terminal decapeptide which is conserved between $G_q\alpha$ and $G_{11}\alpha$ (Strathmann & Simon 1990). This antiserum identifies both polypeptides equally (Mullaney *et al.* 1993a,b). Antiserum I3B was raised against a peptide corresponding to the carboxy-terminal region of $G_{i3}\alpha$, KNNLKECGLY ($G_{i3}\alpha$, 345–354) (Mitchell *et al.* 1989). $G_o\alpha$ was identified by antiserum IO raised against the peptide NLKEDGI SAAKDVK ($G_o\alpha$, 22–35) (Mitchell *et al.* 1989, Mullaney & Milligan 1990) and by antiserum OC (ANNLRGCGLY; $G_o\alpha$ alpha, 345–354) (Mullaney & Milligan 1990).

Electrophoretic resolution and immunoblot analysis

High-resolution urea-SDS-PAGE (12.5% w/v acrylamide/0.0625% w/v bis-acrylamide containing 6 M urea) was carried out overnight at 100 V as described in details before (Mullaney *et al.* 1993b, Svoboda & Milligan 1994, Svoboda *et al.* 1996b). Molecular mass determinations were based on prestained molecular mass markers (Sigma, SDS 7B). After urea-SDS-PAGE, proteins were transferred to nitrocellulose (Schleicher & Schuell, Germany) and the immunoblots were developed with *o*-dianidizine hydrochloride as a substrate for horseradish peroxidase (Mitchell *et al.* 1989). Alternatively, an alkaline phosphatase and nitroblue tetrazolium-based detection protocol was used according to Novotný *et al.* (1995). The developed blots were scanned with a BioRad GS 670 imaging densitometer or a UMAX Astra 610P scanner to enable quantification by the ImageQuant 3.3 program.

RESULTS

$G_q\alpha$ and $G_{11}\alpha$ proteins

PM-enriched fractions of BAT were isolated on sucrose density gradients and resolved by SDS-PAGE on gels containing 6 M urea and a low concentration of bis-acrylamide (see Materials and Methods). This technique had been successfully used for separation and detection of $G_q\alpha$ and $G_{11}\alpha$ proteins in CHO cells (Mullaney *et al.* 1993b, Svoboda & Milligan 1994) and HEK cells (Svoboda *et al.* 1996b). Membrane samples of BAT were compared with those from CHO cells, which were used as positive standards. Under these conditions of electrophoretic resolution, both $G_q\alpha$ and $G_{11}\alpha$ proteins could be unequivocally identified in BAT by direct comparison with corresponding polypeptides present in CHO cells (Fig. 1). Similar results were obtained by comparison with membranes from HEK cells (not shown).

The immunoblot signals of $G_q\alpha$ and $G_{11}\alpha$ detected in membrane samples prepared from cold-acclimated animals were lower than those detected in controls (Table 1) and this decrease was roughly the same for both proteins – 61% and 58% for $G_q\alpha$ and $G_{11}\alpha$ respectively.

$G_{i3}\alpha$ protein

$G_{i3}\alpha$ protein was identified with antiserum I3B (directed against the carboxy-terminal decapeptide of $G_{i3}\alpha$; amino acids 159–168) and an immunoblot

FIGURE 1. G_qα and G₁₁α proteins. Proteins in PM from BAT were resolved on 12·8% acrylamide/0·06% bis-acrylamide/6 M urea gel and the immunoblot signals were detected by CQ antiserum combined with peroxidase-*o*-catechol-based detection. W, control animals, 150 µg protein; C, cold-acclimated animals, 150 µg protein; HM1, clone hm1 of CHO cells, 60 µg protein per lane.

profile obtained with this antiserum in BAT was compared with that detected in rat brain and white adipose tissue. Resolution of I3B-reactive polypeptides in BAT by urea-SDS-PAGE (Fig. 2) revealed two polypeptides. The polypeptide with lower mobility (G_i3α) exhibited in BAT the stronger immunoblot signal (Fig. 2), but in brain microsomes, while lower levels of this protein were noted, the second polypeptide exhibiting the higher mobility represented the dominant I3B-reactive band (G_oα). This profile of the immunoblot data was in accord with results published before

TABLE 1. G_qα and G₁₁α proteins in PM of BAT from control and cold-acclimated hamsters. One hundred and fifty micrograms membrane proteins were resolved on 12·8% acrylamide/0·06% bis-acrylamide/6 M urea gel and the immunoblot signal detected by CQ antibodies. The numbers represent the average of densitometric scans (expressed in arbitrary units) of 12 independent membrane preparations (6 from control and 6 from cold-acclimated animals) ± s.e.m.

	Control	Cold
G _q α	39·3 ± 5·5 (100%)	23·9 ± 2·3 (61%)*
G ₁₁ α	47·1 ± 3·2 (100%)	27·4 ± 2·7 (58%)*

**P* < 0·05, significant effects of cold acclimation (Student's *t*-test).

FIGURE 2. G_i3α protein. Sixty micrograms PM proteins from BAT were subjected to urea-SDS-PAGE (see Materials and Methods) and the immunoblot signals were detected by antiserum I3B combined with alkaline phosphatase. W, controls; C, cold-acclimated; Br, brain, 20 µg protein per lane.

(Milligan 1990) describing cross-reactivity of I3B antiserum with G_oα, but not with G_i1α and G_i2α. The strong I3B-reactive band determined in brain cortex reflects the extremely high content of G_oα proteins in this tissue, which amounts to up to 1% of the total membrane protein (Gierschik *et al.* 1986). Note in this respect that the I3B-reactive polypeptide of higher mobility (G_oα) was not detected in white adipose tissue, where the content of G_oα is very low (Mitchell *et al.* 1989) (see later Fig. 4B). In white adipose tissue, just a single polypeptide was noted with I3B antiserum and the mobility of this band was identical to that of G_i3α identified in BAT (Fig. 4B).

G_oα proteins

In the rat brain cortex, which was used as positive standard of G_oα proteins, the G_oα-specific antiserum IO (internal sequence of G_oα; amino acids 22–35) recognized three bands which might be identified, in order of increasing mobility and using terminology of Goldsmith *et al.* (1988), as G_o*α, G_o1α and G_o2α respectively (compare also with Caufield *et al.* 1994) (Fig. 3). In the BAT, there was no signal corresponding to G_o*α, a very low signal

FIGURE 3. $G_o\alpha$ proteins. Sixty micrograms PM proteins from BAT were subjected to urea-SDS-PAGE and antiserum IO was used for detection of immunoblots. W, controls; C, cold-acclimated; Br, brain, 20 μ g protein per lane.

corresponding to $G_o1\alpha$ and a relatively strong signal with mobility identical to that of $G_o2\alpha$ detected in brain microsomes. These polypeptides could also be detected in rat white adipose tissue (Fig. 4C), where their migration was identical to that of authentic rat brain $G_o1\alpha$ or $G_o2\alpha$. However, the density of $G_o\alpha$ immunoblot signals in white fat membranes was at the detection limit of the method used in this work. This finding is in accord with data published previously (Mitchell *et al.* 1989) showing an extremely low amount of these proteins in white adipose tissue. Simultaneously, the presence of $G_o\alpha$ proteins in BAT represents a genuine characteristics of this tissue, because these proteins were also detected in differentiated brown adipocytes cultivated for 8–9 days in primary cultures (Fig. 5), but were completely absent in non-differentiated pre-adipocytes (day 3–4).

Comparison of the immunoblot data obtained from PM samples isolated from control and cold-acclimated animals demonstrated that the content of these G proteins was decreased by cold acclimation (Table 2).

Mitochondrial contamination

Mitochondrial contamination of PM fractions was estimated as succinate-cytochrome c reductase according to King (1967). The difference between the two types of PM preparations analysed in this study (4 independent preparations) was not statistically significant (26 ± 5 and 31 ± 4 nmol cytochrome c/min per mg for controls and cold-acclimated respectively). This is in accord with data obtained before on other PM-enriched fractions isolated by sucrose density gradients (Svoboda *et al.* 1993, Bouřová *et al.* 1999).

DISCUSSION

Identification of $G_q\alpha$ and $G_{11}\alpha$

In this study, $G_q\alpha$ and $G_{11}\alpha$ proteins were identified in BAT and compared in PM isolated from control and cold-acclimated hamsters. The strategy that was adopted was to utilize quantitative immunoblotting with an antiserum that identifies each of

FIGURE 4. $G\alpha$ proteins in brain, and white and brown adipose tissue. Twenty micrograms brain microsomes (Br), 80 μ g crude membranes from white adipose tissue (WF) or 80 μ g BAT PM (BF) were resolved by 12.8% acrylamide/0.06% bis-acrylamide/6 M urea gel and immunoblotted with CQ (A), I3B (B) or IO (C) antiserum. Alkaline phosphatase was used for development of immunoblots. The results shown are from one representative immunoblot.

FIGURE 5. G_oα proteins in primary cultures of BAT. Crude membranes were prepared from brown fat cells cultivated for 3–4 or 8–9 days in primary culture (see Materials and Methods) and subjected to standard SDS-PAGE on 10% acrylamide/0.26% bis-acrylamide (60 μg protein per lane). The immunoblot signals were detected by antiserum IO combined with alkaline phosphatase-based detection. Br, 20 μg brain microsomes. The results shown are from one representative immunoblot.

these polypeptides equally because it is directed against a sequence that is entirely conserved between these two polypeptides. However, because the predicted molecular masses of murine G_qα and G₁₁α are almost identical, it was necessary to find SDS-PAGE conditions suitable for their resolution, because in 10% acrylamide gel the two polypeptides migrate as one immunologically defined band (not

shown). Using 13% acrylamide gel in the presence of 6 M urea, considerable resolution of these polypeptides was achieved. It was also shown that both proteins are reduced in parallel by cold acclimation of hamster BAT (Table 1).

Thus, the first analysis of the phosphoinositidase C-linked G proteins G_qα and G₁₁α in BAT supports previous evidence for widespread distribution of both these G proteins (Mitchell *et al.* 1991, Mullaney *et al.* 1993a,b), but it remains to be established whether they are coexpressed in individual brown fat cells. The evidence for the functional role of G_q/G₁₁α proteins in BAT is supported by the finding that PT does not prevent the α-adrenergic-stimulated breakdown of phosphoinositides and respiration in BAT (Schimmel & Elliot 1986). The existence of functional phospholipase C- and/or calcium-dependent signalling pathways in BAT has been previously documented (Mohell *et al.* 1984, Nanberg & Putney 1986, Nanberg & Nedergaard 1987, Wilcke & Nedergaard 1989).

Identification of G_i3α and G_oα proteins

The expression of G_i1α and G_i2α proteins in BAT has been clearly documented before (De Pergola *et al.* 1994, Svoboda *et al.* 1996a), but the existence of G_i3α was unclear. In white adipose tissue, G_i3α mRNA was very weakly, if at all, expressed (Watkins *et al.* 1989, Rapiejko *et al.* 1990, Xu *et al.* 1993), but an immunoblot analysis with G_i3α-specific anti-peptide antiserum I3B did reveal the existence of this protein in white adipocytes (Mitchell *et al.* 1989). In this work, using the same antiserum, specific signal of G_i3α could be clearly detected and quantitatively evaluated in BAT of control and cold-acclimated hamsters. Using the same strategy as that applied for identification of G_qα/G₁₁α proteins (high resolution by urea-SDS-PAGE), the two distinct G_oα antiserum-reactive polypeptides were recognized in BAT. The form of G_oα with lower mobility (which we hereafter called G_o1α) was present in low amounts, whereas the form with greater mobility (which we hereafter called G_o2α) represented the dominant immunoreactive polypeptide.

In contrast to our findings, some of the previous reports dealing with white adipocytes did not show the presence of G_oα proteins in these cells (Hinsch *et al.* 1988). This is understandable in view of our own (initial) problems with detection of these low-abundance G proteins in BAT, which were (later) solved by introduction of a more specific and sensitive immunoblot procedure based on alkaline phosphatase (see Materials and Methods). Our

TABLE 2. G_i3α and G_oα proteins in PM from BAT from control and cold-acclimated hamsters. Sixty micrograms membrane proteins were resolved on 12.8% acrylamide/0.06% bis-acrylamide/6 M urea gel and the immunoblot signals detected with I3B or IO antibodies. The numbers represent the average of densitometric scans (expressed in arbitrary units) of six independent membrane preparations (three from control and three from cold-acclimated animals) ± S.E.M.

	Control	Cold
G _i 3α	34.1 ± 2.7 (100%)	19.7 ± 2.3 (58%)*
G _o 1α	32.6 ± 1.8 (100%)	20.3 ± 1.6 (62%)**
G _o 2α	82.3 ± 3.7 (100%)	60.7 ± 2.5 (74%)**

**P<0.01, *P<0.05, significant effects of cold acclimation (Student's *t*-test).

results are similar to those described by Mitchell *et al.* (1989), who detected an extremely weak but significant signal of $G_o\alpha$ in white adipocytes. It thus appears that hamster BAT does indeed express $G_o\alpha$ but in very low quantities in comparison with the other PT-sensitive G proteins.

As brown fat is richly endowed with nerve terminals, an objection could be raised that the presence of $G_{o1}\alpha$ and $G_{o2}\alpha$ might not represent a genuine content of these G proteins in BAT, but rather contamination of membrane fractions by fragments of nerve ending origin. This possibility, however, does not seem to be likely, as the same antisera detected $G_o\alpha$ proteins in differentiated brown adipocytes which were cultivated for 8–9 days in primary cultures (Fig. 5). These proteins were completely absent in non-differentiated pre-adipocytes (day 3–4).

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