Guanine-nucleotide-binding proteins expressed in rat white adipose tissue

Identification of both mRNAs and proteins corresponding to G_i1, G_i2 and G_i3

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Considerable debate has focused on the molecular identity of the guanine-nucleotide-binding proteins (G-proteins) in adipose tissue which can be detected following pertussis-toxin-catalysed ADP-ribosylation [Rapiejko, Northup, Evans, Brown & Malbon (1986) *Biochem. J.* **240**, 35–40; Hinsch, Rosenthal, Spicher, Binder, Gausepohl, Frank, Schultz & Joost (1988) *FEBS Lett.* **238**, 191–196]. We have used a panel of selective anti-peptide antisera which are able to discriminate between the different pertussis-toxin-sensitive G-proteins to assess which of these are expressed in rat adipose tissue. We demonstrate that plasma membranes of rat white adipocytes contain α subunits corresponding to each of G_i1, G_i2 and G_i3. Furthermore, using synthetic oligonucleotides complementary to unique regions of each of the three polypeptides, we demonstrate that the mRNAs for the three G-protein α subunits can also be detected in adipose tissue.

INTRODUCTION

Receptor-mediated inhibition of adenylate cyclase activity is attenuated by pretreatment with pertussis toxin [1]. This occurs because the toxin catalyses an NAD⁺-dependent ADP-ribosylation of a 41 kDa polypeptide [2]. As such, this polypeptide was identified as the α subunit of the inhibitory G-protein of the adenylate cyclase cascade (G_i) [2]. ADP-ribosylation occurs at a cysteine residue located four amino acids from the C-terminus and the modification appears to be sufficient to prevent productive interactions between receptors and G_i.

Both from protein purification protocols [3,4] and from analysis of cDNA clones [5,6], it has become clear that a considerable number of pertussis-toxin-sensitive G-proteins exist. These include G_o, a G-protein thought to be involved in receptor-mediated alterations in Ca²⁺-channel function [4], as well as G-proteins named G_i1, G_i2 and G_i3 [5]. The nomenclature of the 'G_i-like' G-proteins is related more to the high degree of sequence similarity between them than to an assessment of their functional roles, which, despite good evidence to indicate that G_i3 is able to act as a regulator of K⁺ channels [7], are poorly defined. As the α -subunits of each of these proteins are close to 40 kDa in size, it is essentially impossible to identify adequately the individual gene products using pertussis-toxin-catalysed [³²P]ADP-ribosylation followed by electrophoretic separation and autoradiography. To address this problem, a number of investigators have generated polyclonal antisera against purified G-protein fractions [8–10]. Whilst the use of such antisera has produced more information, the realization that the individual pertussis-toxin-sensitive G-

proteins are extremely similar implies that these antisera might not display a sufficiently high degree of specificity to unequivocally identify the individual species.

Information taken from cDNA clones corresponding to the various G-proteins has allowed the generation of anti-peptide antisera against sequences which are unique to the various gene products [11–14]. Here we use a series of highly selective anti-(G-protein) anti-peptide antisera, together with oligonucleotide probes to the mRNAs, to assess the expression of G-proteins in membranes of rat adipocytes. This tissue has been shown previously to express multiple forms of pertussis-toxin-sensitive G-proteins, but the molecular identity of these species has not been demonstrated unequivocally.

MATERIALS AND METHODS

Membrane preparation for immunoblotting

Plasma membranes of white adipocytes derived from rat epididymal fat pads were prepared as described previously [15]. Rat glioma C6 BU1 cells were grown in tissue culture and a crude membrane pellet was prepared as previously described in detail [16]. A crude membrane fraction was prepared from rat cerebral cortex in a similar fashion to that from the glioma cells. Membrane protein was suspended in 20 μ l of 10 mM-Tris/HCl/0.1 mM-EDTA, pH 7.5. An aliquot (10 μ l) of 50 mM-dithiothreitol/5% (w/v) SDS was added and the samples were placed in a boiling water bath for 10 min. Following cooling, 10 μ l of 100 mM-N-ethylmaleimide was added and the samples left at room temperature for 30 min. The alkylation reaction was terminated by the addition of

Abbreviation used: PAGE, polyacrylamide-gel electrophoresis.

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Table 1. (a) Generation and specificities of antisera used, (b) comparison of amino acid sequences of the C-terminal decapeptides of the α subunits of pertussis-toxin-sensitive G-proteins, (c) comparison of amino acids 159–168 of $G_{i1}\alpha$ with equivalent regions of other pertussis-toxin-sensitive G-proteins

(a) Antiserum SG1 cannot discriminate between $G_{i1}\alpha$ and $G_{i2}\alpha$ as the sequence used to generate the antiserum is conserved between the two polypeptides. This antiserum also identifies both rod (TD1) and cone (TD2) variants of transducin α ; however, as these are restricted to photoreceptor-containing tissues, then this antiserum can be used to identify $G_{i1}\alpha$ or $G_{i2}\alpha$ in all other locations. (see [12] for discussion of this point).

Anti-serum	Peptide employed	Corresponding G-protein sequence	Antiserum identifies:
(a)			
SG1	KENLKDCGLF	TD1 341–350	TD1, TD2, G_{i1} , G_{i2}
I1A	LDRIAQPNYI	G_{i1} 159–168	G_{i1}
LE2	LERIAQSDYI	G_{i2} 160–169	G_{i2}
LE3	LERIAQSDYI	G_{i2} 160–169	G_{i2}
I3B	KNNLKECGLY	G_{i3} 345–354	G_{i3}
IM1	NLKEDGISAAKDVK	G_o 22–35	G_o
(b)			
G_{i1}	KNNLKDCGLF	G_{i1} (159–168)	LDRIAQPNYI
G_{i2}	KNNLKDCGLF	G_{i2} (160–169)	LERIAQSDYI
G_{i3}	KNNLKECGLY	G_{i3} (159–168)	LDRISQTNFY
G_o	ANNLRGCGLY	G_o (160–169)	LDRIGAADYQ
TD1	KENLKDCGLF	TD1 (155–164)	LRLVTPGYV
TD ₂	KENLKDCGLF	TD2 (159–168)	LDRITAPDYL

Laemmli buffer prior to addition of the samples to SDS/PAGE.

Immunological analysis

The generation and specificities of the various antisera used in this study are defined in Table 1. Confirmation of the specificities of previously uncharacterized antisera is provided in the text. Each antiserum was produced in a New Zealand White rabbit with a conjugate of a synthetic peptide and keyhole-limpet haemocyanin (Calbiochem). The details of this process have previously been recorded in [12].

Membrane samples were resolved by SDS/PAGE [12.5% acrylamide, 0.06% bisacrylamide (w/v)] in 20 cm $\times$ 20 cm slab gels overnight at 100 V. Proteins were transferred to nitrocellulose (Schleicher & Schuell) and blocked for 2 h at 25 °C with 3% (w/v) skimmed milk (Marvel) in Tris-buffered saline (TBS) (20 mM-Tris/HCl, pH 7.5/500 mM-NaCl) or with 3% gelatin in TBS under similar conditions except that the incubation was at 37 °C. Primary antiserum (1:200 dilution unless otherwise noted) in 1% Marvel/TBS (or with 1% gelatin under equivalent conditions) was then added and left overnight. The primary antiserum was then removed and the blot was washed extensively with distilled water followed by washes with TBS containing 0.1% (v/v) Tween-20 (TTBS) and then TBS. Secondary antiserum (donkey anti-rabbit IgG coupled to horseradish peroxidase) (Scottish Antibody Production Unit, Wishaw, Scotland) (1:200 dilution) in 1% Marvel (or 1% gelatin)/TBS was

Table 2. Generation of oligonucleotide probes for the identification of mRNAs corresponding to the α subunits of G_{i1} , G_{i2} and G_{i3}

mRNA identified	Oligonucleotide sequence used
$G_{i1}\alpha$	5'-GCTGTCCTTCCACAGTCTCTTTATG-ACGCCGGC-3'
$G_{i2}\alpha$	5'-ATGGTCAGCCCAGAGCCTCCGGAT-GACGCCCA-3'
$G_{i3}\alpha$	5'-GCCATCTCGCCATAAACGTTTAAT-CAGCCTGC-3'

added and left for 3 h. Removal of the secondary antiserum was followed by the same series of extensive washes of the nitrocellulose as detailed following removal of the primary antiserum. *o*-Dianisidine hydrochloride (Sigma) was employed as the substrate for detection of the antibody complex as previously described [14].

Pertussis-toxin-catalysed ADP-ribosylation

Membranes of rat adipocytes were ADP-ribosylated as described in [17] using thiol-preactivated pertussis toxin (Porton Products, Porton Down, Wiltshire, U.K.) and [³²P]NAD (New England Nuclear). Samples were resolved, following alkylation, in identical SDS/PAGE slabs to those used for the immunoblotting experiments. The gels were then dried and autoradiography performed for 48 h using Kodak X-O-Mat X-ray film.

Generation of oligonucleotide probes

Synthetic 33-mer oligodeoxynucleotides (Table 2) (generated on an Applied Biosystems DNA synthesizer) were complementary to bases of the mRNAs encoding amino acids 125–135 of the α subunits of each of G_{i1} , G_{i2} and G_{i3} . This region corresponds to one of the highly divergent areas of the coding sequence of these G-proteins as defined by analysis of rat cDNA clones [5]. Labelling of probes at the 5' end was achieved with ³²P (6000 Ci/mmol, Amersham International) and T4 polynucleotide kinase (Boehringer Mannheim). Incorporation of radioactivity averaged 7×10^8 d.p.m./ μ g of probe.

Detection of mRNA species corresponding to G_{i1} , G_{i2} and G_{i3}

Male Sprague–Dawley rats were stunned by a sharp blow to the head and killed by cervical dislocation. Epididymal fat pads were excised and rinsed in ice-cold 0.9% NaCl. The tissue was then ground to a fine powder in liquid N₂ and subsequently homogenized in 6 ml of GIT buffer (4 M-guanidinium thiocyanate solution/25 mM-sodium acetate, pH 6.0/120 mM-2-mercaptoethanol) [18] followed by extrusion through 18 and 23G needles. The homogenate was then centrifuged (10 000 g, 10 min) to remove particulate material, and the supernatant was layered over 6 ml of caesium chloride buffer (5.7 M-caesium chloride/25 mM-sodium acetate, pH 6) in polyallomer ultracentrifuge tubes (Beckman). Samples were centrifuged overnight in a Beckman SW40 rotor (170 000 g, 20 h, 20 °C).

Total RNA was recovered from the bottom of the tubes, resuspended in GIT buffer, extracted once against each of phenol/chloroform (1:1, v/v) and chloroform,

Fig. 1. Pertussis-toxin-catalysed ADP-ribosylation of G-proteins in rat tissues

ADP-ribosylation of membranes from rat (1) glioma C6 BU1 cells (50 μ g), (2) white adipocytes (20 μ g), and (3) cerebral cortex (20 μ g) was performed with [32 P]NAD $^{+}$ and thiol-activated pertussis toxin as described in the Materials and methods section. Samples were resolved in SDS/PAGE slabs, which after drying were autoradiographed. Tentative identities of the various pertussis-toxin-sensitive polypeptides are based on their relative electrophoretic mobility and previous immunological studies [12,13,32].

and finally precipitated with ethanol. The concentration of precipitated RNA was determined by absorbance at 260 nm.

Northern blotting

Total RNA was denatured by incubation at 65 $^{\circ}$ C with 2 M-formaldehyde and 50 % deionized formamide. It was resolved in 1 % agarose gels containing 2.2 M-formaldehyde before transfer to 'Hybond N' nylon filters (Amersham) [19]. Filters were prehybridized at 42 $^{\circ}$ C for 4 h in hybridization solution [50 % deionized formamide, 5 $\times$ SSPE (1 $\times$ SSPE = 150 mM-NaCl/10 mM-sodium dihydrogen phosphate/1 mM-EDTA pH 7.4), 5 $\times$ Denhardt's solution (5 $\times$ Ficoll 400, bovine serum albumin and polyvinylpyrrolidone, each at 1 mg/ml)/0.1 % SDS/100 μ g of denatured salmon testes DNA/ml] and were then hybridized (42 $^{\circ}$ C, 16 h) in hybridization solution containing the relevant oligodeoxynucleotide probes (5 $\times$ 10 6 c.p.m./ml).

The blots were washed to a final stringency of

Fig. 2. Identification of both G $_i$ 1 and G $_i$ 2 in rat adipocytes

Membranes from (1) rat glioma C6 BU1 cells (30 μ g), (2) rat adipocytes (30 μ g) and (3) rat cerebral cortex (100 μ g) were resolved and immunoblotted as described in the Materials and methods section. The blot was developed using antiserum SG1 (1:200 dilution) as the primary reagent. This antiserum identifies both G $_i$ 1 and G $_i$ 2 equally (see Table 1).

0.5 $\times$ SSC (1 $\times$ SSC = 150 mM-NaCl/15 mM-tri-sodium citrate, pH 7.0)/0.1 % SDS (55 $^{\circ}$ C, 30 min). Autoradiographic localization of the bound probes was performed by exposure to presensitized X-ray film at -80 $^{\circ}$ C. Alternatively, filters were stained with Methylene Blue to locate rRNA species [20].

RESULTS

Pertussis-toxin-catalysed [32 P]ADP-ribosylation of rat adipocyte plasma membranes caused the incorporation of radioactivity into two distinct and well-resolved polypeptides with apparent sizes close to 40 kDa (Fig. 1). The band with greater mobility through the gel co-migrated with the major pertussis-toxin-sensitive substrate of rat glioma C6 BU1 cells, which has previously been shown to correspond to G $_i$ 2, whilst the polypeptide with lower mobility co-migrated with the major form of G $_i$ from rat cerebral cortex (G $_i$ 1). To define the molecular identity of these species we used a series of anti-peptide antisera of known specificity in membranes from each of rat adipocytes, brain and glioma C6 BU1 cells.

Antiserum SG1 identifies G $_i$ 1 and G $_i$ 2 equally, as these two proteins have an identical sequence in the region used to generate the antiserum. This antiserum does not identify G $_i$ 3 (see later). Antiserum SG1 identified two polypeptides in rat adipocyte membranes of 40 and 41 kDa (Fig. 2). In the same experiment only a single polypeptide which migrated identically with the 40 kDa polypeptide from rat adipocytes was identified in membranes of C6 BU1 cells (G $_i$ 2). In contrast, however, in membranes from rat cerebral cortex, two polypeptides were identified which co-migrated with the two polypeptides of rat fat. The relative staining of 40 to 41 kDa

Fig. 3. Independent identification of both G_1 and G_2 in rat adipocytes

(a) Membranes from rat (1) C6 BU1 cells (60 μ g), (2) adipocytes (60 μ g) and (3) cerebral cortex (100 μ g) were treated as in Fig. 2, except that the primary antiserum was LE3 (1:120 dilution). A single polypeptide of 40 kDa (G_2) was identified in all tissues. (b) Membranes of rat (1) glioma C6 BU1 cells (200 μ g), (2) adipocytes (200 μ g) and (3) cerebral cortex (200 μ g) were resolved and immunoblotted using antiserum I1A (1:60 dilution) as primary antiserum, as described in the Materials and methods section. This antiserum identified a single polypeptide of 41 kDa (G_1) in adipocyte and cerebral cortex membranes but not C6 BU1 membranes. (c) Membranes of rat (1) cerebral cortex (200 μ g), (2) adipocytes (100 μ g) and (3) glioma C6 BU1 cells (100 μ g) were treated as above and the immunoblot developed using a mixture of antisera LE3 (1:200) and I1A (1:60). This Figure demonstrates that the two antisera identify mutually exclusive polypeptides. Both cerebral cortex and adipocytes express both G_1 and G_2 ; C6 BU1 cells expresses G_2 but not detectable quantities of G_1 .

polypeptides ($40 < 41$) was as previously noted for the relative proportions of G_2 to G_1 in brain [4].

To further define these conclusions we employed anti-peptide antisera which are able to discriminate between G_1 and G_2 . Antiserum LE3 identifies only G_2 [4]. This antiserum identified but a single polypeptide of 40 kDa in rat adipocytes, glioma C6 BU1 cells and cerebral cortex membranes, thus demonstrating that the 40 kDa polypeptide was indeed G_2 (Fig. 3a). Further, the relative intensity of staining of this polypeptide in the three tissues by antiserum LE3 was similar to that noted for the 40 kDa polypeptide identified by antiserum SG1 (compare Figs. 2 and 3a). Antiserum I1A identifies only G_1 . Immunoblotting with this antiserum identified a single polypeptide of 41 kDa which co-migrated in membranes of rat cerebral cortex and rat adipocytes (Fig. 3b). No immunoreactivity to this antiserum was observed in C6 BU1 membranes. Immunoblotting these tissues with a mixture of antisera LE3 and I1A confirmed that these two antisera identified mutually exclusive polypeptides (Fig. 3c).

Antiserum I3B is directed against the C-terminal decapeptide of $G_{i3\alpha}$. This antiserum identified a single polypeptide of 41 kDa in membranes of both rat fat and C6BU1 cells (Fig. 4) but failed to identify a polypeptide of this size in rat cerebral cortex. Thus, this immunoreactivity in rat adipocytes was not a reflection of a slight cross-reactivity of the antiserum with G_1 , which migrates with a similar, but slightly higher, apparent molecular mass. A mixture of antisera LE2 and I3B identified a doublet of polypeptides in both C6 BU1 cells and adipocytes, demonstrating that these antisera identified

mutually exclusive polypeptides. The upper band co-migrated with the single polypeptide identified by antiserum I3B ($G_{i3\alpha}$) (Fig. 4b).

To detect mRNAs coding for the α subunits of each of G_1 , G_2 and G_3 , we probed Northern blots of total RNA from rat adipose tissue with synthetic oligonucleotides (Table 2) constructed to hybridize selectively with relevant mRNAs. Each probe hybridized with a single band on Northern blots; the G_1 probe to an RNA of 3.45 kb, G_2 to an RNA of 2.35 kb and the G_3 probe to an RNA of 3.4 kb (Fig. 5). These values agree well with mRNA lengths published elsewhere [5].

We also performed immunoblots of both rat cerebral cortex and fat with the antiserum IM1 which we have previously shown to be specific for G_o [14]. Whilst the signal, using this antiserum, was extremely weak in rat adipocyte membranes, it was possible to detect a polypeptide which migrated exactly as did rat brain G_o . This polypeptide was only present in adipocyte membranes at about 1% of the level observed in brain membranes (results not shown). It thus appears that rat adipocytes do indeed express G_o , but in very low quantities in comparison with the other pertussis-toxin-sensitive G-proteins.

DISCUSSION

At least two pertussis-toxin-sensitive G-proteins are expressed in membranes of rat adipocytes [21–23]. The debate relating to the nature of these polypeptides developed because at the time of this observation it had been noted that brain also expressed a number of different

Fig. 4. Detection of the α subunit of G_3 in rat adipocytes

(a) Membranes of rat (1) adipocytes (150 μ g) and (2) C6 BU1 cells (100 μ g) were resolved and immunoblotted as in Fig. 3 using antiserum I3B (1:200). A single polypeptide of 41 kDa ($G_{3\alpha}$) was identified in both tissues. (b) To confirm that antiserum I3B did not cross-react with G_2 , membranes of rat (1) C6 BU1 cells (100 μ g) and (2) adipocytes (100 μ g) were resolved and immunoblotted with (A) antiserum LE2, (B) a mixture of antisera I3B and LE2 or (C) antiserum I3B. A doublet containing polypeptides of 40 and 41 kDa was observed with the mixture but only single polypeptides were detected with the individual antisera. In a similar experiment with rat cerebral cortex, I3B did not identify a 41 kDa polypeptide, indicating that this antiserum does not identify G_1 (results not shown).

G-proteins [3,24,25], of which at least two (now named G_{i1} and G_o) were present in high concentrations and could be shown to be immunologically distinct [8,25,26]. It was presumed likely that the two distinct polypeptides in adipose tissue would also be these forms. The recognition that those polypeptides identified as ' G_i ' in brain and neutrophils [27] and rat glioma cells [28] were immunologically distinct indicated that further molecular forms of pertussis-toxin-sensitive G-proteins must be expressed.

Whilst a number of studies employing immunological methods have confirmed the expression of ' G_i -like' G-proteins in adipocytes [29,30], conflicting results have been obtained using immunological probes for G_o . Using a polyclonal antiserum raised against purified G_o , Malbon and coworkers have argued strongly for G_o being expressed in rat adipocytes [22,30]. In contrast, Hinsch *et al.* [21] failed to detect G_o in rat adipose tissue when using an antiserum raised against a peptide which corresponded to a sequence close to the N-terminus of G_o and has been used by a number of investigators to

Fig. 5. Detection of mRNAs corresponding to G_1 , G_2 and G_3 in rat adipose tissue

mRNA coding for G-protein α subunits was identified as described in the Materials and methods section. Each lane contained 10 μ g of total RNA from rat fat. Lanes 1, 2 and 3 were hybridized with synthetic oligodeoxynucleotides complementary to G_1 , G_2 and G_3 respectively. The positions of the 18 and 28 S rRNA species are indicated. Autoradiograms were exposed for 4 days.

generate highly selective anti- G_o antibodies [11,14,31]. Here, using an anti-peptide antiserum (IM1), which interacts strongly with rat brain G_o , we were able to detect only an extremely weak signal in rat adipocyte membranes of a polypeptide which migrated identically in our gel system with authentic rat brain G_o .

We have thus attempted to clearly define the nature of the pertussis-toxin-sensitive G-proteins expressed in rat adipocytes by using a series of anti-peptide antisera which display selectivity for each of the ' G_i -like' pertussis toxin-sensitive G-proteins which have currently been characterized. Rat brain expresses high levels of G_{i1} and G_o and lower levels of G_{i2} [12]. Rat glioma C6 BU1 cells express high levels of G_{i2} [12] and lower levels of G_{i3} . We have demonstrated that the two most prevalent pertussis-toxin-sensitive G-proteins in rat adipocytes correspond immunologically to G_{i1} and G_{i2} , both by their reactivity with specific anti-peptide antisera and because both are identified by an antiserum incapable of distinguishing between these two gene products. Further evidence of the molecular identity of these polypeptides is that the α subunits G_{i1} and G_{i2} found in rat adipocytes migrate identically with the equivalent forms both in rat cerebral cortex and in rat glioma C6 BU1 cells, systems in which the G-protein populations have previously been well characterized [4,13,32]. Pertussis-toxin-catalysed ADP-ribosylation also identified polypeptides with the expected mobilities of G_{i1} and G_{i2} as the forms present in the greatest concentrations. In addition to these two forms, we have also detected the presence of $G_{i3\alpha}$ in rat adipocytes and in rat glioma cells with a specific

antipeptide antiserum (I3B) directed against the C-terminal decapeptide of $G_{i3\alpha}$. This polypeptide was present in barely detectable quantities in rat cerebral cortex. This is consistent with the inability to detect a pertussis-toxin-sensitive G-protein in rat brain which migrates in 2-dimensional gels at a position equivalent to protein expressed from a G_{i3} cDNA [4]. As antiserum I3B does not recognize the C-terminus of G_{i2} (Fig. 4b) then it follows that it does not identify G_{i1} because G_{i1} and G_{i2} have identical sequences in the region used to generate the antiserum. Our ability to detect each of these specific 'G_i-like' G-protein α subunits is consistent with our ability to detect mRNAs corresponding to the α subunits of each of G_{i1} , G_{i2} and G_{i3} in rat adipose tissue.

Theoretically, any of these G-proteins may be responsible for transducing receptor-generated signals which are attenuated by pretreatment of adipocytes with pertussis toxin, including receptor-mediated inhibition of adenylate cyclase and stimulation of inositol phosphate generation [22]. A major challenge will now be to assign roles to each of these G-proteins.

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