Characterization of GTP-binding proteins in Golgi-associated membrane vesicles from rat adipocytes

Annette SCHÜRMANN,* Walter ROSENTHAL,† Günther SCHULTZ† and Hans G. JOOST*‡

*Institut für Pharmakologie und Toxikologie der RWTH Aachen, Wendlingweg 2, D5100 Aachen, Germany, and

†Institut für Pharmakologie der Freien Universität Berlin, D1000 Berlin 33, Germany

We have previously reported that guanine nucleotides inhibit glucose transport activity reconstituted from adipocyte membrane fractions. In order to further investigate the hypothetical involvement of guanine-nucleotide-binding proteins (GTP-binding proteins) in the regulation of insulin-sensitive glucose transport activity, we studied their subcellular distribution in adipocytes treated or not with insulin. Adipocytes were homogenized and fractionated to yield plasma membranes (PM) and a Golgi-enriched fraction of intracellular membranes (low-density microsomes, LDM). In these membrane fractions, total guanosine 5'-[γ -³⁵S]thio]triphosphate ([³⁵S]GTP[S]) binding, α - and β -subunits of heterotrimeric G-proteins, proto-oncogenes Ha-ras and K-ras, and 23–28 kDa GTP-binding proteins were assayed. The levels of α_s and α_i (the α -subunits of G_s and G_i) were approx. 8-fold lower in LDM than in PM; β -subunits, Ha-ras and K-ras were not detectable in LDM. Total GTP[S]-binding sites and 23–28 kDa GTP-binding proteins were present in LDM in approximately the same concentrations as in PM. Insulin gave rise to the characteristic translocation of glucose transporters, but failed to alter the subcellular distribution of any of the GTP-binding proteins. Fractionation of the LDM on a discontinuous sucrose gradient revealed that α_s and α_i , as detected with antiserum against a common peptide sequence (α_{common}), and the bulk of the 23–28 kDa G-proteins sedimented at different sucrose densities. None of the GTP-binding proteins co-sedimented with glucose transporters. Furthermore, the inhibitory effect of GTP[S] on the reconstituted transport activity was lost in the peak fractions of glucose transporters partially purified on the sucrose gradient. These data indicate that LDM from adipocytes contain several GTP-binding proteins in discrete vesicle populations. However, the intracellular GTP-binding proteins are not tightly associated with the vesicles containing the glucose transporter.

INTRODUCTION

The heterotrimeric guanine-nucleotide-binding proteins (G-proteins) transduce receptor-initiated signals to a variety of effector proteins (Birnbaumer, 1990) including enzymes (Levitzky, 1986) and ion channels (Hescheler *et al.*, 1987; Rosenthal *et al.*, 1988; Birnbaumer, 1991). Furthermore, GTP-binding proteins appear to be involved in the translocation of proteins through the Golgi stack (Melancon *et al.*, 1987), and have been suggested to participate in the regulation of glucose transport activity in adipocytes (Joost *et al.*, 1985; Kuroda *et al.*, 1987). Many different types of receptors, including several receptors for peptide hormones, have been shown to interact with G-proteins (Birnbaumer, 1990). Since the insulin-stimulated activation of phosphodiesterase appears to be mediated by a G-protein (Heyworth *et al.*, 1983), it has been suggested that insulin receptors control a G-protein-dependent signalling chain.

Insulin regulates glucose transport in adipocytes, mainly through translocation of glucose transporters from an intracellular compartment to the plasma membrane (Cushman & Wardzala, 1980; Suzuki & Kono, 1980). Involvement of G-proteins in this process has been suggested on the basis of recent data showing that guanosine 5'-[γ -thio]triphosphate (GTP[S]) produced an insulin-like translocation of glucose transporters in permeabilized adipocytes (Baldini *et al.*, 1991). Lipolytic or antilipolytic agents modulate the intrinsic activity of glucose transporters rather than their concentration in the plasma membrane (Joost *et al.*, 1986). Based on the finding that the effects of catecholamines on glucose transport activity in adipocytes were independent of changes in the adenylate cyclase activity (Joost *et al.*, 1985; Kuroda *et al.*, 1987), it has been

suggested that the glucose transport activity is regulated by a direct interaction of G-proteins with the glucose transporter. This hypothesis has been tested by us in experiments designed to study the effects of guanine nucleotides on the transport activity in a cell-free system (Schürmann *et al.*, 1989). Glucose transport activity was reconstituted from adipocyte membrane fractions and assayed in the presence of guanine nucleotides under conditions unlikely to increase cyclic AMP levels. It was found that guanine nucleotides inhibited the reconstituted glucose transport activity, provided that the cells had been treated with insulin prior to the preparation of membranes. Surprisingly, the effect of the guanine nucleotides was not restricted to the plasma membranes, but was also observed in a Golgi-enriched intracellular membrane fraction (low-density microsomes, LDM). These microsomes contain the glucose transporters that are translocated to the plasma membrane (PM) in response to insulin (Cushman & Wardzala, 1980; Suzuki & Kono, 1980).

In the present study, we further tested the presumed involvement of GTP-binding proteins in the regulation of insulin-sensitive glucose transport. We characterized the GTP-binding proteins in the intracellular microsomes of adipocytes having specific antisera, and tested the possibility that insulin might produce a subcellular redistribution of GTP-binding proteins similar to that of the glucose transporter. Along similar lines, catecholamines have recently been shown to induce internalization of G-proteins (Haraguchi & Rodbell, 1990). Furthermore, we fractionated the intracellular membrane vesicles by density gradient centrifugation in an attempt to identify a GTP-binding protein as a constitutive component of the vesicles containing glucose transporters. Finally, we demonstrated that the effect of GTP[S] on glucose transport activity in LDM is not

Abbreviations used: GTP[S], guanosine 5'-[γ -thio]triphosphate; PM, plasma membranes; LDM, low-density microsomes.

‡ To whom correspondence should be addressed.

preserved after separation of the vesicles on a sucrose gradient. The present data show that G-protein α -subunits and small GTP-binding proteins (23–28 kDa) are present in distinct vesicle populations of the intracellular microsomes from adipocytes, but none of these proteins appears to be a component of the vesicles containing glucose transporters.

MATERIALS AND METHODS

Cell preparation, incubation of adipocytes and preparation of membrane fractions

Male rats (170–200 g; Wistar strain; fed *ad libitum*) were used throughout. The epididymal adipose tissue was removed and digested with collagenase (Type 1; Cooper Biochemical) according to Rodbell (1964) with modifications as described previously (Weber *et al.*, 1988). Adipose cells obtained from 20–30 rats were incubated at 37 °C in two polyethylene beakers, each containing a total volume of 20 ml [resulting in a cell concentration of $(1.5\text{--}2) \times 10^6$ cells/ml]. All incubations were carried out in a KRBH buffer (Weber *et al.*, 1988), pH 7.4, containing 10 mM- NaHCO_3 , 30 mM-Hepes, 2.5 mM-glucose, 200 nM-adenosine and 5% (w/v) BSA (Cohn Fraction V). Insulin (crystalline pig zinc insulin, courtesy of Hoechst AG, Frankfurt, Germany) was added to a final concentration of 8 nM. After 30 min, cells were washed once with 45 ml of TES homogenization buffer (20 mM-Tris, 1 mM-EDTA and 255 mM-sucrose) and homogenized with a Potter–Elvehjem grinder (A. H. Thomas Scientific; cat. no. 3431–E25; specific clearance of 0.15 mm). After homogenization, PM and LDM fractions were prepared by differential centrifugation as previously described in detail (Simpson *et al.*, 1983; Weber *et al.*, 1988). In some experiments the LDM were further separated on a discontinuous sucrose gradient (0.1–1.5 M). Microsomes from 20 rats (approx. 2 mg of protein) were layered on to the gradient and were centrifuged for 60 min at 90 000 g (21 000 rev./min; rotor SW27). Fractions were collected with the aid of a hypodermic needle inserted into the bottom of the tube. The separated vesicles were spun down at 200 000 g (90 min) and resuspended in 10 mM-Tris buffer, pH 7.4.

Assay of glucose transport activity

Glucose transport in plasma membrane vesicles was determined under equilibrium exchange conditions as previously described in detail (Joost *et al.*, 1988). Glucose transport activity reconstituted into lecithin liposomes was assayed essentially as described by Robinson *et al.* (1982), with modifications described previously (Schürmann *et al.*, 1989).

Antisera

The following antisera against peptides corresponding to partial sequences of the respective proteins were employed: (1) an antiserum (Hinsch *et al.*, 1988) against a peptide sequence present in most G-protein α -subunits [α_{common} , sequence in one-letter code: (C)GAGESGKSTIVKQMK], (2) an antiserum (Offermans *et al.*, 1990) against a peptide specific for α_1 -subunits [(C)NLREDGEKAAREV], (3) an antiserum against a peptide corresponding to the sequence of α_s -subunits [IQRMHLRQ-YELL], (4) an antiserum (Hinsch *et al.*, 1989) against a peptide sequence present in all β -subunits [β_{common} , (C)EGNVRVS-RELIGHTGY], and (5) an antiserum against the C-terminal sequence of the glucose transporter GLUT4 [STELEYLGP-DEND]. A monoclonal antibody against c-K-ras (code Ab-1) was purchased from Dianova, Hamburg, Germany. Antiserum against c-Ha-ras was a gift from Dr. K. Aktories, Universität des Saarlandes, Homburg/Saar, Germany.

Immunoblotting

Samples of the membrane fractions (40 μg of protein) were separated by SDS/PAGE and transferred to nitrocellulose membranes (BA85, pore size 0.45 μm ; Schleicher & Schuell, Dassel, Germany) with a semi-dry blotting apparatus (Sartoblot II; Sartorius, Göttingen, Germany). The membranes were blocked overnight by incubation with buffer containing 10 mM-Tris, 0.05% (w/v) Tween 20 and 150 mM-NaCl, and were incubated with antiserum at a dilution of 1:200 for 2 h at room temperature. After three washes with buffer containing BSA (1%, w/v), the nitrocellulose membranes were incubated with ^{125}I -labelled Protein A (0.1 $\mu\text{Ci}/\text{ml}$) for 2 h, thoroughly washed and autoradiographed for 1–4 days.

Labelling of small GTP-binding proteins on nitrocellulose with GTP[S]

Binding of GTP[S] to proteins adsorbed to nitrocellulose membranes was assayed according to a previously published method (Bhullar & Haslam, 1987; Lapetina & Reep, 1987). Briefly, samples of 50 μg of membrane proteins were separated by SDS/PAGE (12% polyacrylamide) and electrophoretically transferred on to nitrocellulose membranes. The transfer was carried out at 150 mA for 16 h in a buffer (pH 8.8) containing Tris (25 mM), glycine (192 mM) and methanol (20%, v/v). Immediately after the transfer, the nitrocellulose membranes were washed twice in buffer (pH 7.5) containing Tris (50 mM), MgCl_2 (100 μM) and Tween 20 (0.1%, w/v). [^{35}S]GTP[S] (1 $\mu\text{Ci}/15\text{ ml}$) was added in the same buffer, and binding was allowed to proceed at room temperature for 90 min. The nitrocellulose membranes were washed five times with fresh buffer, dried and autoradiographed for 2–7 days.

Specific binding of GTP[S] to total membranes

Specific binding of GTP[S] to total membranes was assayed using a modification of a previously published protocol (Northup *et al.*, 1982). Samples of 30–50 μg of membrane protein were incubated with 30 nCi of [^{35}S]GTP[S] (specific radioactivity 1500 Ci/mmol) in the presence of unlabelled GTP[S] or other nucleotides as indicated in a buffer (pH 8.0) containing Tris (20 mM), NaCl (100 mM), EDTA (1 mM), MgCl_2 (10 mM), dithiothreitol (1 mM) and Triton X-100 (0.1%, w/v) in a total volume of 100 μl at 30 °C for 60 min. In some experiments, insulin (16 nM) was added in the presence of MnCl_2 (5 mM) and ATP (100 μM). The incubation was terminated by addition of 1 ml of ice-cold buffer (pH 8.0) containing Tris (20 mM), NaCl (100 mM) and MgCl_2 (25 mM), and the samples were immediately filtered by suction through nitrocellulose filters (Sartorius G.m.b.H., Göttingen, Germany; pore size 0.2 μm) and washed four times with 1 ml of fresh buffer. Radioactivity on the filters was determined by scintillation counting in a water-compatible scintillation cocktail (Instagel; Packard Instruments, Frankfurt, Germany). The binding curves were evaluated with the IBM-PC version of the LIGAND (SCAFIT) program (Munson & Rodbard, 1980) (courtesy of Dr. P. J. Munson, National Institutes of Health, Bethesda, MD, U.S.A.) on the basis of a single site model.

RESULTS

Isolated adipocytes were incubated with or without insulin and were fractionated to yield PM and a Golgi-enriched preparation of intracellular vesicles (LDM). Using this well-defined procedure (Simpson *et al.*, 1983; Weber *et al.*, 1988), we routinely obtained plasma membranes exhibiting a large effect of insulin on stereospecific glucose uptake into the vesicles. The basal

transport of D-glucose in PM was 5.6 ± 0.9 pmol/s per mg of protein, and this was increased to 76.2 ± 15 pmol/s per mg of protein after treatment of cells with insulin. Furthermore, the well-recognized effect of insulin on the subcellular distribution of glucose transporters (Cushman & Wardzala, 1980; Suzuki & Kono, 1980) became apparent after solubilization and reconstitution of the glucose transport activity into lecithin liposomes. The reconstituted glucose transport activity was decreased by insulin in the LDM (basal, 2.64 ± 0.5 nmol/s per mg of protein; insulin, 1.1 ± 0.1 nmol/s per mg) and was increased in the plasma membranes (basal, 0.25 ± 0.1 nmol/s per mg of protein; insulin, 0.76 ± 0.2 nmol/s per mg).

Fig. 1 illustrates the immunochemical detection of several GTP-binding proteins in adipocyte membrane fractions. The α -subunits of the heterotrimeric G-proteins G_s and G_i (Fig. 1a) were detected with an antiserum against a highly conserved peptide sequence (α_{common}). The antiserum reacted with bands at 45 kDa and 42 kDa, previously identified as α -subunits of G_s , and with a somewhat broader band at 40 kDa, identified as two isoforms of G_i (Hinsch *et al.*, 1988; Schürmann *et al.*, 1989). In contrast to several non-specific bands at higher and lower molecular masses, these bands were fully suppressed by incubation of antiserum with the α_{common} peptide (results not shown). Both α -subunits were present in PM and in LDM, and insulin failed to alter their subcellular distribution. In order to

Fig. 1. Subcellular distributions of G-protein α - and β -subunits and of proto-oncogenes Ha-ras and K-ras in adipocytes

Isolated adipocytes were incubated with or without insulin, homogenized and fractionated as described in the Materials and methods section. PM (BP, PM from basal cells; IP, PM from insulin-treated cells) and LDM (BL, LDM from basal cells; IL, LDM from insulin-treated cells) were separated by SDS/PAGE, transferred on to nitrocellulose membranes and immunoblotted with antiserum against G-protein α -subunits (α_{common} , a), β -subunits (β_{common} , b), Ha-ras (c) and K-ras (d).

Table 1. Distribution of GTP-binding proteins in membrane fractions from control and insulin-stimulated adipocytes

Isolated adipocytes were incubated in the presence or absence of insulin, and membrane fractions were prepared as described in the Materials and methods section. BPM, basal plasma membranes; IPM, plasma membranes from insulin-treated cells; BLD, basal LDM; ILDM, LDM from insulin-treated cells. Data represent means $\pm$ S.E.M. from at least four different membrane preparations. Total GTP[S]-binding sites were calculated from the binding curves presented in Fig. 2. GTP[S] binding to blotted proteins was determined by cutting and counting of radioactivity in the labelled bands (Fig. 3). The immunoreactivity of G-protein α -subunits was determined by cutting and counting of radioactivity in the bands labelled with ^{125}I -Protein A (Fig. 4). The data were normalized to the total label in all GTP-binding proteins from a set of membranes.

	BPM	IPM	BLD	ILD
Total GTP[S]-binding sites (pmol/mg of protein)	231 $\pm$ 55	216 $\pm$ 37	166 $\pm$ 43	130 $\pm$ 21
GTP[S] binding to blotted proteins (c.p.m./total c.p.m.)				
23/24 kDa	12.4 $\pm$ 3.4	13.9 $\pm$ 2.0	30.8 $\pm$ 2	27.8 $\pm$ 2.4
26 kDa	9.2 $\pm$ 2.2	11.2 $\pm$ 1.6	16.2 $\pm$ 1.8	14.7 $\pm$ 0.9
28 kDa	12.8 $\pm$ 1.0	15.4 $\pm$ 1.7	18.9 $\pm$ 3.5	17.4 $\pm$ 2.8
Immunoreactivity of G-proteins α -subunits (c.p.m./total c.p.m.)				
45 kDa (α_s)	16.7 $\pm$ 2.4	16.3 $\pm$ 1.6	1.9 $\pm$ 0.6	2.4 $\pm$ 0.5
40 kDa (α_i)	60.9 $\pm$ 5.0	60.1 $\pm$ 6.9	8.4 $\pm$ 0.7	7.9 $\pm$ 1.4
β -subunit	97.6 $\pm$ 0.6	96.2 $\pm$ 1.3	2.6 $\pm$ 0.6	3.8 $\pm$ 1.4
Proto-oncogenes				
Ha-ras	98.0 $\pm$ 2.0	98.9 $\pm$ 0.4	2.1 $\pm$ 1.9	1.2 $\pm$ 0.4
K-ras	96.2 $\pm$ 0.7	96.5 $\pm$ 1.2	3.8 $\pm$ 0.7	3.5 $\pm$ 1.2

assess the relative abundance of the G-protein α -subunits in the membrane fractions, the amount of ^{125}I -Protein A incorporated into the respective bands was determined and compared in a series of independent membrane preparations (Table 1). This comparison indicated that the LDM contained approx. 15% of the amount of G-protein α -subunits detected in the PM.

The β -subunits of the heterotrimeric G-proteins were detected with antiserum against a highly conserved peptide sequence (β_{common}). As is illustrated in Fig. 1(b), the serum reacted with a 36 kDa band corresponding to the β -subunit. In contrast with the subcellular distribution of the α -subunits, virtually no β -subunits were detected in the LDM fraction. The quantification of ^{125}I -Protein A incorporation into the immunoreactive bands (Table 1) indicated that the LDM contained less than 4% of the amount of β -subunit present in the PM. It appears reasonable to assume that this amount represents a contamination of LDM with PM, which has been assessed at 5% by previous investigators (Simpson *et al.*, 1983). Like the β -subunits of the heterotrimeric G-proteins, the GTP-binding proteins Ha-ras (Fig. 1c) and K-ras (Fig. 1d) were exclusively detected in the PM fraction.

In order to assess the subcellular distribution of total GTP-binding proteins, specific binding of [^{35}S]GTP[S] was assayed in PM and LDM fractions. In preliminary experiments we established the specificity of these sites for guanine triphosphates (results not shown): GTP[S] and GTP (100 μM) inhibited binding of the tracer by more than 90%. GDP (100 μM) inhibited [^{35}S]GTP[S] binding by 50%, whereas GMP and ATP (100 μM) failed to inhibit the binding of the tracer. Fig. 2 illustrates a competition curve of binding of [^{35}S]GTP[S] and unlabelled GTP[S] in PM and LDM from basal and insulin-stimulated

Fig 2. Specific binding of GTP[S] in adipocyte PM and LDM

Adipocyte PM (a) or LDM (b) were incubated with 50000 c.p.m. of $[^{35}\text{S}]\text{GTP[S]}$ and the indicated concentrations of unlabelled GTP[S] for 60 min at 30 °C as described in the Materials and methods section. ●, Membranes from basal cells; ○, membranes from insulin-stimulated cells. The data are means $\pm$ S.E.M. from five different membrane preparations.

adipocytes. Scatchard analysis of the binding curves indicated that the LDM contained a large amount of GTP[S]-binding sites, only about 30 % less sites than in the PM (Table 1). Furthermore, treatment of cells with insulin failed to alter the number or affinity of GTP-binding sites in PM and LDM [K_D in basal plasma membranes, 111 ± 32 nM (mean $\pm$ S.E.M.); in plasma membranes from insulin-treated cells, 89 ± 25 nM; in basal LDM, 74 ± 21 nM; in LDM from insulin-treated cells, 63 ± 19 nM].

The GTP-binding proteins detected in the LDM were further characterized by labelling with $[^{35}\text{S}]\text{GTP[S]}$ after separation by SDS/PAGE and transfer on to nitrocellulose membranes. As illustrated in Fig. 3, three major GTP-binding proteins with apparent molecular masses of 23–24, 26 and 28 kDa were labelled with this technique. These proteins were present in both PM and LDM (Fig. 3a); binding of the tracer to these proteins was fully inhibited by unlabelled GTP (Fig. 3b). In order to assess the relative abundance of the 23–28 kDa GTP-binding proteins, we determined and compared the radioactivity incorporated into the respective bands (Table 1). PM and LDM contained comparable amounts of each of the small GTP-binding proteins (Table 1). Insulin failed to alter the binding of $[^{35}\text{S}]\text{GTP[S]}$ to the 23–28 kDa GTP-binding proteins (Fig. 3, Table 1).

Fig 3. Labelling of small GTP-binding proteins with GTP[S] in adipocyte membrane fractions

Adipocyte PM and LDM were separated by SDS/PAGE (12 % gels) and proteins were transferred on to nitrocellulose membranes. The membranes were incubated with 1 μCi of $[^{35}\text{S}]\text{GTP[S]}$ in the absence (a) or the presence (b) of unlabelled GTP[S] (100 μM), washed, and autoradiographed. Lane 1, basal PM; lane 2, plasma membranes from insulin-treated cells; lane 3, basal LDM; lane 4, LDM from insulin-treated cells; lane 5, membranes from rat brain.

LDM represent a heterogeneous population of vesicles which can be further separated by sedimentation on a density gradient. In order to assess the distribution of the vesicles containing GTP-binding proteins and to compare it with that of vesicles containing glucose transporters, LDM were layered on a sucrose gradient and centrifuged for 60 min at 90000 g . Fractions were collected and assayed by immunoblotting with antiserum against the α_{common} peptide, against a peptide sequence present in all α_i -subunits, $\alpha_{i\text{-common}}$, and against a sequence specific for α_s (Fig. 4). With the exception of the $\alpha_{i\text{-common}}$ antiserum, which reacted with an unidentified protein at 45 kDa, the antisera labelled only bands in the 40–45 kDa range, identified as G-protein α -subunits. Fig. 4 illustrates the distribution of the G-protein α -subunits on the density gradient. The α -subunits of G_i , as detected with the anti- α_{common} serum (Fig. 4a) or with the anti- $\alpha_{i\text{-common}}$ serum (Fig. 4b), sedimented as a relatively sharp peak at a sucrose concentration of 0.6–0.8 M. In contrast, the two α -subunits of G_s detected with an antiserum against the C-terminus of α_s sedimented over a broad range of the sucrose gradient (0.2–0.8 M-sucrose). Surprisingly, the anti- α_{common} serum cross-reacted only with the α_s isoforms sedimenting at the low sucrose density (0.2–0.4 M), and failed to detect those sedimenting at 0.4–0.8 M-sucrose. Thus the LDM appear to contain four different isoforms of α_s , distinguishable by their molecular mass, their cross-reactivity with the α_s and the α_{common} antisera, and their sedimentation on the sucrose gradient. Attempts to label the α_s isoforms in the LDM by cholera toxin-catalysed ADP-ribosylation were unsuccessful, presumably because the microsomes lack ADP-ribosylation factor (results not shown).

Fig. 5 compares the distributions of 23–28 kDa GTP-binding proteins, glucose transporters and G-protein α -subunits on a sucrose density gradient. The 23–28 kDa GTP-binding proteins were present in a broad range of the vesicle population, whereas the α -subunits of G_s and G_i detected with the α_{common} antiserum formed two peaks at different densities. As already shown in Fig. 4, the vesicles containing glucose transporters formed a peak at a sucrose density of 0.2–0.6 M. None of the GTP-binding proteins

Fig 4. Immunoblot detection of G-protein α -subunits and glucose transporter GLUT4 in LDM separated on a sucrose density gradient

LDM from insulin-treated adipocytes were centrifuged on a discontinuous sucrose gradient as described in the Materials and methods section. Fractions were isolated and separated by SDS/PAGE, transferred on to nitrocellulose membranes, and immunoassayed with the α_{common} antiserum (a), or with specific sera against α_i (b), α_s (c) and GLUT4 (d). For comparison, samples of PM membranes (IPM) and LDM (ILD) were run on the same gel (a–c).

Fig 5. Distribution of the LDM vesicles containing the glucose transporter and GTP-binding proteins on a sucrose density gradient

LDM from insulin-treated adipocytes were centrifuged on a discontinuous sucrose gradient as described in the Materials and methods section. Fractions were separated by SDS/PAGE, transferred on to nitrocellulose membranes and immunoassayed with the α_{common} antiserum ($\circ$, α_i ; $\bullet$, α_s) and with serum against the glucose transporter GLUT4 ($\blacktriangle$). Small GTP-binding proteins (23–28 kDa, $\square$) were labelled with [35 S]GTP[S]. The radioactivity incorporated into the labelled bands was determined, and was calculated as a percentage of that found in the respective bands of crude microsomes which were run on the same gel.

Fig 6. Distribution of the reconstitutable glucose transport activity from LDM on a sucrose density gradient, and its inhibition of GTP[S]

LDM from insulin-treated adipocytes were centrifuged on a discontinuous sucrose gradient as described in the Materials and methods section. Fractions were isolated, solubilized and reconstituted into lecithin liposomes. Glucose transport was assayed in the presence or absence of 100 μ M-GTP[S]. Control data (absence of GTP[S]) were calculated and presented as a percentage of the glucose transport activity assayed in crude microsomes; the inhibitory effect of GTP[S] was calculated as a percentage of the decrease in glucose transport rates in the control samples. $\bullet$, Glucose transport activity in the absence of GTP[S]; $\blacktriangle$, relative inhibitory effect of GTP[S].

appeared to be specifically associated with the vesicle population containing glucose transporters.

We have previously shown that GTP[S] inhibits glucose transport activity reconstituted from LDM of insulin-treated adipocytes by 40–60% (Schürmann *et al.*, 1989). If this effect was mediated by a G-protein associated with the vesicles containing glucose transporters, it should be preserved after partial purification of the transporters on the sucrose gradient. As illustrated in Fig. 6, however, the further purification of the LDM on the sucrose gradient abolished the inhibitory effect of GTP[S] in the peak fractions of glucose transport activity. GTP[S] produced a 45% inhibition of the transport activity in the unfractionated LDM (not shown in Fig. 6), whereas no significant inhibitory effect was detectable in the peak fractions of transport activity sedimenting at 0.4 and 0.6 M-sucrose (Fig. 6). Furthermore, the Figure demonstrates that GTP[S] still inhibited the transport activity in the vesicles sedimenting at 0–0.2 M-sucrose. Thus it is tempting to speculate that an inhibitory, GTP-dependent, factor is present in those vesicles sedimenting at 0–0.2 M-sucrose. However, it has to be noted that the transport activities reconstituted from these fractions (0–0.2 M-sucrose) were close to the level of detectability. Therefore we cannot fully discount the possibility that the fractionation of the vesicles has abolished the inhibitory effect of GTP[S] by removing a soluble factor or by degradation of the inhibitory factor.

DISCUSSION

The present data indicate that a fraction of Golgi-enriched intracellular membranes (LDM) prepared from adipocytes contains significant amount of α -subunits of G_s and G_i . Cross-contamination of the LDM with PM cannot account for the presence of these α -subunits, since their abundance clearly exceeded that of three other PM proteins (β -subunits, c-Ha-ras and c-K-ras). Surprisingly, the intracellular G-proteins appeared to lack β -subunits, which were not detectable in the LDM. The $\beta\gamma$ complex is required for the receptor-catalysed activation of α -subunits by exchange of GDP for GTP (Birnbaumer, 1990). Thus an intracellular α -subunit can only be activated after

translocation to the PM. It is unlikely, therefore, that the G-protein α -subunits present in the LDM operate an exclusively intracellular component of the cellular machinery. The intracellular G-proteins, locked in their GDP-binding state, may instead be part of an endocytic/exocytic pathway.

The present data indicate that insulin, in spite of inducing a large translocation of transporters from the LDM to the PM, does not alter the subcellular distribution of any of the GTP-binding proteins investigated. The possibility has to be considered that the anticipated effect of insulin has not been detected because the portion of translocated G-protein is much smaller than the total amount of this G-protein. Previous studies have shown that about 25 pmol of glucose transporters/mg of protein is translocated in response to insulin (Cushman & Wardzala, 1980; Joost *et al.*, 1988). In the present study, the abundance of total GTP-binding sites (Table 1) was about 200 pmol/mg of protein, probably including heterotrimeric G-proteins as well as the low-molecular-mass G-proteins. It would be difficult to detect a small effect of insulin, e.g. a change by 10 pmol/mg, against such a large background. However, the abundance of the individual G-protein subtypes is much smaller: adipocyte membranes contain approx. 40 pmol of 39 kDa G-protein α -subunit/mg (Ros *et al.*, 1988). Thus it appears reasonable to conclude that the individual G-proteins are present only in moderate excess over the transporter. Consequently, an effect of insulin on their subcellular distribution should be detectable with subtype-specific antibodies (Fig. 1) and in the ligand blot with GTP[S] (Fig. 3), unless only a small portion of the transporters forms a complex with a G-protein. However, we cannot fully discount the possibility that a subtype of the rapidly growing family of G-proteins which was not assayed in the present study might be translocated together with the transporter in response to insulin.

Based on the finding that GTP[S] inhibits the reconstituted glucose transport activity in insulin-treated adipocytes, we have previously suggested that glucose transporters interact with GTP-binding proteins in an insulin-dependent manner (Schürmann *et al.*, 1989). The present study failed to demonstrate a tight association between GTP-binding proteins and the glucose transporter, as would have been shown by co-purification or co-translocation of these proteins (Figs. 1 and 5). However, the present findings do not necessarily rule out the interaction of glucose transporters and G-proteins at the level of the PM or in the intracellular compartment. It is conceivable that they interact only in the appropriate cellular compartment, and dissociate upon translocation of either component. The present data render it unlikely, however, that the inhibitory effect of GTP[S] on glucose transport activity is mediated by a heterotrimeric G-protein, since LDM, in which the effect of GTP[S] was clearly visible, were virtually devoid of β -subunits. Furthermore, at present we can only speculate on the mechanism of the insulin-dependence of the effect of GTP[S]. Insulin has previously been described to alter G-protein function (Heyworth & Houslay, 1983; Heyworth *et al.*, 1985), and it is conceivable that such an alteration is necessary for the inhibitory effect of GTP[S] on transport activity. It appears unlikely that the effect of GTP[S] requires an insulin-induced alteration of the transporter, e.g. translocation of the transporters to the PM, since a comparable inhibitory effect of GTP[S] was observed in both LDM and PM fractions.

In addition to the heterotrimeric G-proteins, a group of smaller GTP-binding proteins (19–28 kDa) has previously been identified (Lapetina & Reep, 1987; Bhullar & Haslam, 1987; Hall, 1990). These small GTP-binding proteins have been detected in plasma membranes (Lapetina & Reep, 1987; Bhullar & Haslam, 1987), cytosolic preparations (Lapetina & Reep, 1987), secretory vesicles of adrenal chromaffin cells (Burgoyne &

Morgan, 1989) and rough endoplasmic reticulum of hepatocytes (Lanoix *et al.*, 1989). In the present study, three small GTP-binding proteins (23–28 kDa) were identified by labelling of the membrane proteins with [³⁵S]GTP[S] on nitrocellulose membranes. These proteins were almost as abundant in the LDM as in the PM, and it is tempting to speculate that they are responsible for the inhibitory effect of GTP[S] on glucose transport activity. They appear to be similar, if not identical, to the GTP-binding proteins previously described in platelets, brain and other tissues (Lapetina & Reep, 1987; Bhullar & Haslam, 1987; Lanoix *et al.*, 1989; Burgoyne & Morgan, 1989). The function of these GTP-binding proteins has not yet been fully elucidated, but evidence has been presented that they participate in the transport of proteins through the Golgi stack (Melancon *et al.*, 1987) and in exocytic/endocytic processes (Chavrier *et al.*, 1990). It has recently been demonstrated that high concentrations of GTP[S] induce an insulin-like translocation of glucose transporters in permeabilized adipocytes (Baldini *et al.*, 1991). On the basis of the present data, it appears unlikely that this effect is mediated by heterotrimeric G-proteins associated with the LDM, since these G-proteins lack the $\beta\gamma$ complex. Thus the GTP[S]-mediated translocation of glucose transporters might instead be mediated by one of the small GTP-binding proteins.

We have previously characterized the G-protein α -subunits in adipocytes with specific antibodies derived from peptides corresponding to peptide sequences of the α -subunits G_i , G_s and G_o (Hinsch *et al.*, 1988). Adipocytes contain two different α -subunits of G_s (42 and 45 kDa), probably generated by alternative splicing (Bray *et al.*, 1986), and two G_i species (40 kDa). The present data provide circumstantial evidence suggesting that even more isoforms of α_s are expressed in adipocytes. When LDM were separated on a sucrose gradient, the two isoforms of α_s detected with serum against its C-terminus were sedimented over a broad range of the sucrose gradient (Fig. 4). Surprisingly, only the portion of these G-proteins sedimenting at a lower sucrose density cross-reacted with the α_{common} antiserum. This heterogeneity of the α_s subunit in the LDM cannot be entirely explained by the previously described alternative splicing of the α_s mRNA (Bray *et al.*, 1986), since all four known splicing variants should contain the α_{common} sequence encoded by exon 1 and should therefore cross-react with the α_{common} antiserum. Thus it can only be speculated that the discrepant immunoreactivities of the α_s isoforms in the LDM reflect a post-translational modification or the presence of other, as yet unidentified, splicing products. Furthermore, we cannot fully discount the possibility that the antisera cross-reacted with another subtype of the rapidly growing family of G-proteins (Simon *et al.*, 1991).

We thank Dr. K. Aktories for the gift of the antiserum against c-Ha-ras, and Dr. K.-D. Hinsch and K. Spicher for the antisera against the heterotrimeric G-proteins. The skilful assistance of C. Schmitz-Salue is gratefully acknowledged. The results are part of the Ph.D. thesis of A.S. This study was supported by the Deutsche Forschungsgemeinschaft.

REFERENCES

- Baldini, G., Hohman, R., Charron, M. J. & Lodish, H. F. (1991) *J. Biol. Chem.* **266**, 4037–4040
- Bhullar, R. P. & Haslam, R. J. (1987) *Biochem. J.* **245**, 617–620
- Birnbaumer, L. (1990) *Annu. Rev. Pharmacol. Toxicol.* **30**, 675–705
- Birnbaumer, L. (1991) *Biol. Reprod.* **44**, 207–224
- Bray, P., Carter, A., Simons, C., Guo, V., Puckett, C., Kamholz, J., Spiegel, A. & Nirenberg, M. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 8893–8897
- Burgoyne, R. D. & Morgan, A. (1989) *FEBS Lett.* **245**, 122–126
- Chavrier, P., Parton, R. G., Hauri, H. P., Simons, K. & Zerial, M. (1990) *Cell* **62**, 317–329
- Cushman, S. W. & Wardzala, L. J. (1980) *J. Biol. Chem.* **255**, 4758–4762

- Hall, A. (1990) *Science* **249**, 635–640
- Haraguchi, K. & Rodbell, M. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1208–1212
- Hescheler, J., Rosenthal, W., Trautwein, W. & Schultz, G. (1987) *Nature (London)* **325**, 445–447
- Heyworth, C. M. & Houslay, M. D. (1983) *Biochem. J.* **214**, 547–552
- Heyworth, C. M., Wallace, A. V. & Houslay, M. D. (1983) *Biochem. J.* **214**, 99–110
- Heyworth, C. M., Whetton, A. D., Wong, S., Martin, B. R. & Houslay, M. D. (1985) *Biochem. J.* **228**, 593–603
- Hinsch, K. D., Rosenthal, W., Spicher, K., Binder, T., Gausepohl, H., Frank, R., Schultz, G. & Joost, H. G. (1988) *FEBS Lett.* **238**, 191–196
- Hinsch, K. D., Tychowiecka, I., Gausepohl, H., Frank, R., Rosenthal, W. & Schultz, G. (1989) *Biochim. Biophys. Acta* **1013**, 60–67
- Joost, H. G., Göke, R. & Steinfeld, H. J. (1985) *Biochem. Pharmacol.* **34**, 649–653
- Joost, H. G., Weber, T. M., Cushman, S. W. & Simpson, I. A. (1986) *J. Biol. Chem.* **261**, 10033–10036
- Joost, H. G., Weber, T. M. & Cushman, S. W. (1988) *Biochem. J.* **249**, 155–161
- Kuroda, M., Honnor, R. G., Cushman, S. W., Londos, C. & Simpson, I. A. (1987) *J. Biol. Chem.* **262**, 245–253
- Lanoix, J., Roy, L. & Paiement, J. (1989) *Biochem. J.* **262**, 497–503
- Lapetina, E. G. & Reep, B. R. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2261–2265
- Levitzky, A. (1986) *Physiol. Rev.* **66**, 819–854
- Melancon, P., Glick, B. S., Malhotra, V., Weidman, P. J., Serafini, T., Gleason, M. L., Orci, L. & Rothman, J. E. (1987) *Cell* **51**, 1053–1062
- Munson, P. J. & Rodbard, D. (1980) *Anal. Biochem.* **107**, 220–239
- Northup, J. K., Smigel, M. D. & Gilman, A. G. (1982) *J. Biol. Chem.* **257**, 11416–11423
- Offermans, S., Schäfer, R., Hoffmann, B., Bombien, E., Spicher, K., Hinsch, K. D., Schultz, G. & Rosenthal, W. (1990) *FEBS Lett.* **260**, 14–18
- Robinson, F. W., Blevins, T. L., Suzuki, K. & Kono, T. (1982) *Anal. Biochem.* **122**, 10–19
- Rodbell, M. (1964) *J. Biol. Chem.* **239**, 375–380
- Ros, M., Northup, J. K. & Malbon, C. C. (1988) *J. Biol. Chem.* **263**, 4362–4368
- Rosenthal, W., Hescheler, J., Hinsch, K. D., Spicher, K., Trautwein, W. & Schultz, G. (1988) *EMBO J.* **7**, 1627–1633
- Schürmann, A., Rosenthal, W., Hinsch, K. D. & Joost, H. G. (1989) *FEBS Lett.* **255**, 259–264
- Simon, M. I., Strathmann, M. P. & Gautam, N. (1991) *Science* **252**, 802–808
- Simpson, I. A., Yver, D. R., Hissin, P. J., Wardzala, L. J., Karnieli, E., Salans, L. B. & Cushman, S. W. (1983) *Biochim. Biophys. Acta* **763**, 393–407
- Suzuki, K. & Kono, T. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2542–2545
- Weber, T. M., Joost, H. G., Simpson, I. A. & Cushman, S. W. (1988) in *Receptor Biochemistry and Methodology. Insulin Receptors Part B: Biological Responses, and Comparison to the IGF-I Receptor* (Kahn, C. R. & Harrison, L., eds.), pp. 171–187, Alan R. Liss Inc., New York

Received 12 August 1991/1 November 1991; accepted 18 November 1991