Possible role for gp160 in constitutive but not insulin-stimulated GLUT4 trafficking: dissociation of gp160 and GLUT4 localization

Anthony FILIPPIS*, Stella CLARK† and Joseph PROIETTO*[1]

*The University of Melbourne, Department of Medicine, Royal Melbourne Hospital, Parkville 3050, Victoria, Australia and †Baker Medical Research Institute, Prahran 3181, Victoria, Australia

GLUT4-containing vesicles are constantly cycling in both basal and insulin-stimulated states. Our previous studies have shown that basal cycling of GLUT4 is impaired under conditions of high glucose or glucosamine and, as a consequence, GLUT4 is retained intracellularly in low-density microsomes [Filippis A., Clark, S., and Proietto, J. (1997) Biochem. J. **324**, 981–985]. In addition to GLUT4 itself, a major protein component of GLUT4-containing vesicles is a glycoprotein of M_r 160000 (gp160). In all studies so far published gp160 has been co-localized with GLUT4 under all conditions. In this study, we show that retention of GLUT4 in low-density microsomes (enriched in Golgi apparatus) is associated with a decrease in gp160 levels in this compartment. A concomitant increase of gp160 in high-density microsomes (enriched in endoplasmic reticulum), demonstrates for the first time a dissociation in the localization of gp160 and GLUT4. Despite the marked decrease in gp160 levels in the GLUT4-containing compartment, insulin-stimulated translocation was normal, while little gp160 appeared in the plasma membrane in response to insulin. The retention of gp160 in the high-density microsomes is apparently not due to a change in the glycosylation state of gp160 as measured by [^{3}H]mannose incorporation. It is concluded that, in rat adipocytes, gp160 is not required for insulin-stimulated translocation, but may be necessary for constitutive trafficking of the GLUT4-containing vesicle.

INTRODUCTION

We have recently shown that in rat adipocytes an increased flux of both glucose and glucosamine through the hexosamine biosynthesis pathway inhibits glucose transport and leads to the internalization of the glucose transporter, GLUT4, basally [1]. However, despite a similar decrease in glucose transport, GLUT4 translocation to the plasma membrane was normal under insulin-stimulated conditions [1]. The mechanism underlying basal GLUT4 retention is unknown.

In both the basal and insulin-stimulated state, GLUT4 is continually recycling between the plasma membrane and intracellular pool [2]. In resting fat cells, GLUT4 is found predominantly compartmentalized in intracellular low-density microsomes (LDM) as a component protein of a specific intracellular vesicle. This vesicle, referred to as 'the GLUT4-containing vesicle', is a unique compartment containing a number of associated proteins (aside from GLUT4), such as gp160 [3], SCAMPs (secretory compartment-associated membrane proteins) [4,5], VAMPs (vesicle-associated membrane proteins) [6], PtdIns 4-kinase [7] and Rab4 (GTPase of Rab family) [8]. Upon insulin stimulation of fat cells, the GLUT4-containing vesicles, together with some of the above-mentioned associated proteins are translocated from the intracellular compartment to the cell surface (for review see [9]). Of these proteins, only the glycoprotein of M_r 160000 (gp160) [3,10] is restricted to GLUT4-containing vesicles and cycles to and from the cell surface in a manner indistinguishable from GLUT4 [3]. gp160 is found exclusively in rat fat and muscle cells [11] and may be considered as a marker for insulin-stimulated glucose transport [11]. In all studies published so far gp160 and GLUT4 have been co-localized under all conditions, raising the possibility that gp160 may play a role in insulin-mediated GLUT4 translocation.

In this study we investigate whether modification of gp160 is responsible for the changes previously observed in GLUT4 trafficking in the presence of either glucosamine or elevated glucose concentrations [1].

EXPERIMENTAL

Materials

Collagenase (Type 5) was purchased from Worthington Biochemicals (Frehol, NJ, U.S.A.). Human insulin was purchased from Novo-Nordisk A/S (North Rocks, NSW, Australia). Dinonyl phthalate oil was from Fluka Chemie AG (Buchs, Switzerland). 2-Deoxy-D-[U-^{14}C]glucose, [2-^{3}H]adenosine monophosphate, uridine diphospho-D-[U-^{14}C]galactose and D-[2-^{3}H]mannose were obtained from Amersham International (Amersham, Bucks, U.K.). 3-*O*-Methyl-D-[U-^{14}C]glucose and enhanced chemiluminescence reagents were obtained from Dupont NEN (Boston, MA, U.S.A.). PVDF membrane was purchased from Schleider and Schuell (Dassel, Germany). Leupeptin was purchased from Boehringer Mannheim GmbH (Germany). Beckman Ready-SOLV EP scintillant was purchased from Beckman Instruments Inc. (Galway, Ireland). The gp160 antibody was a generous gift from Professor P. Pilch (Boston University Medical Centre, Boston, MA, U.S.A.). Bradford protein assay reagent, acrylamide and Dowex 2-X8, 200–400 mesh (Cl^- form) were purchased from Bio-Rad (Richmond, CA, U.S.A.). BSA (Fraction V) was purchased from ICN (Costa Mesa, CA, U.S.A.). Adenosine deaminase, adenosine monophosphate, *N*-acetylglucosamine, UDP-galactose, mannose,

Abbreviations used: LDM, low-density microsomes; HDM, high-density microsomes; TBS, Tris-buffered saline.

[1] To whom correspondence should be addressed.

Table 1 5′-Nucleotidase and UDP-galactose:*N*-acetylglucosamine galactosyltransferase activities in rat adipocytes

5′-Nucleotidase and UDP-galactose:*N*-acetylglucosamine galactosyltransferase activities expressed as a percentage of their respective highest specific activity observed within each preparation. Each value represents the mean ± S.E.M. of three separate membrane preparations. PM, plasma membranes.

Fraction	5′-Nucleotidase (%)	Galactosyltransferase (%)
Homogenate	24.2 ± 1.8	23.8 ± 2.8
PM	100	24.1 ± 3.3
LDM	21.8 ± 2.2	100

PMSF and all other reagent-grade chemicals were purchased from Sigma (St. Louis, MO, U.S.A.).

Animals

Male Sprague-Dawley rats (approx. 200 g) were purchased from Monash University Animal Facility (Clayton, Victoria, Australia) and were fed standard laboratory chow (Barastoc Products, Clark King, Victoria, Australia).

Adipocyte preparation

Rats weighing approx. 200 g were killed by CO_2 gassing and cervical dislocation. Rat epididymal fat pads were collected and adipocytes obtained by collagenase digestion (1.5 mg/ml), using the procedure of Rodbell [12] as modified by Kelada et al. [13]. Briefly, adipocytes were isolated by shaking finely minced tissue in polypropylene flasks at 37 °C for 45 min in Krebs–Ringer Hepes buffer, pH 7.4, containing 1.15 mM Ca^{2+}/3 % BSA/5 mM glucose. Adipocytes were separated from fibrous tissue by sieving and then washed twice in equal volumes of Krebs–Ringer Hepes buffer, followed by a 30 min equilibration period. The number of cells in each preparation was determined using a haemocytometer.

Adipocyte pre-incubation

Following digestion, cells (1×10^6 cells/ml) were distributed into 25 ml polypropylene flasks (10 ml/flask) with the adipocytes floating on a thin layer of buffer in an atmosphere of carbogen (5 % CO_2/95 % O_2) with shaking (100 rpm), at 37 °C. Studies were performed under both basal and submaximal (0.7 nM) insulin-stimulated conditions. Pre-incubation for 2 h was initiated by the addition of either (i) low glucose (5 mM), or (ii) high glucose (30 mM) in the presence of insulin (0.7 nM), or (iii) glucosamine (3 mM). Glucose alone has a slow entry rate into adipocytes and insulin is necessary to promote glucose entry into the cells. To keep the basal adenosine levels low, adenosine deaminase (2 U/ml) was added to each of the pre-incubation conditions. Following the 2 h pre-incubation, adipocytes were washed three times in equal volumes of glucose and insulin-free Krebs–Ringer Hepes buffer. The washing procedures effectively removed all extracellular insulin, glucose or glucosamine and allowed the glucose transport system to deactivate to basal levels. After this time, cells from each incubation condition were washed and fractionated to yield LDM, high-density microsomes (HDM), plasma membranes or a total membrane fraction, as described below.

Glucose-uptake assays

The rate of glucose uptake under basal and insulin-stimulated adipocytes has been measured using either [^{14}C]2-deoxyglucose or [^{14}C]-3-*O*-methylglucose. 2-Deoxyglucose is readily taken up by adipocytes and undergoes hexokinase-mediated phosphorylation on entering the cell interior. Although hexokinase activity is normally not rate-limiting in adipocytes, the argument can be made that under certain experimental conditions phosphorylation could become rate-limiting and thus confound accurate measurements of glucose transport activity. To address this potential problem, additional studies were performed using 3-*O*-methylglucose which cannot undergo phosphorylation.

2-Deoxyglucose was measured as previously described [1,13]. After washing, adipocytes were resuspended in Krebs–Ringer Hepes buffer (2.5 ml) at pH 7.4 and incubated in the absence and presence of various insulin concentrations with 5.0 mM glucose in a shaking waterbath for 30 min at 37 °C. Adipocytes were incubated with 2-deoxy-D-[U-^{14}C]glucose (0.1 mM at 0.25 μCi/tube) for 10 min. To confirm that changes in glucose uptake were due to changes in transport and not phosphorylation, 3-*O*-methylglucose transport was measured in some experiments as described previously [14,15]. Briefly, aliquots (200 μl) of adipocytes (1×10^6 cells/ml) were mixed with 500 μl of 3-*O*-methylglucose (final concentration, 0.5 mM) and 0.1 μCi of 3-*O*-methyl-D-[U-^{14}C]glucose. The assay was commenced by the addition of cells and terminated by the addition of 1 mM ice-cold phloretin. Cells were incubated for 10 s to measure basal and 2 s to measure insulin-stimulated uptake rates. Cells were separated from buffer by centrifugation through oil (14250 ***g***; 1 min) and cell-associated radioactivity was determined using a liquid-scintillation counter.

Subcellular fractionation of adipocytes

The various cell fractions were prepared from incubated adipocytes by a modification of the standard technique of differential ultracentrifugation as employed by Kelada et al. [13]. Following incubation, cells were homogenized (14 strokes at setting 7, equivalent to 1400 rpm) on a Janke and Kunkel homogenizer (Staufen, Germany), in a buffer containing 20 mM Tris (pH 7.4)/1 mM EDTA/0.25 M sucrose. All subsequent steps were carried out at 4 °C using this buffer. The homogenate was placed into polycarbonate tubes and centrifuged in a Beckman JA-20 rotor for 2 min at 6000 ***g*** to obtain a fat-free cell homogenate. The cell homogenate was then centrifuged as previously described [1,13]. The various membrane fractions obtained were all resuspended in homogenization buffer containing two protease inhibitors (400 μM PMSF/10 mg/ml leupeptin) and stored at −70 °C.

Membrane characterization

5′-Nucleotidase assay

The 5′-nucleotidase assay was used as a marker of plasma membrane enrichment [16]. The protocol followed a modification of the method employed by Avruch and Wallach [17]. The 5′-nucleotidase assay was performed in a microfuge tube, in a volume of 100 μl. The assay mixture contained 50 μl of 100 mM Tris (pH 7.4)/10 mM $MgCl_2$/1 mM AMP as a sodium salt/tracer [2-^{3}H]AMP/50 μl of membrane suspension. The assay mixture was incubated at 37 °C for a period of 30–60 min. Control samples contained no enzyme. The reaction was terminated by

Table 2 5′-Nucleotidase activities under various incubation conditions in rat adipocytes

5′-Nucleotidase activities were measured in three separate membrane preparations, as described in the legend to Table 1. PM, plasma membranes.

Fraction	Basal (%)	Insulin (%)	High glucose (%)	Glucosamine (%)
Homogenate	19.4 ± 3.2	15.3 ± 2.1	21.4 ± 5.4	18.9 ± 3.9
PM	100	100	100	100
LDM	28.7 ± 1.9	25.1 ± 4.5	26.2 ± 3.4	23.5 ± 2.6

the addition of 20 μl of 250 mM $ZnSO_4$. Protein and unhydrolysed AMP were precipitated by the addition of 20 μl of 250 mM $Ba(OH)_2$. The adenosine present in each sample remained in the supernatant. Each sample was then centrifuged at full speed in a Beckman Microfuge B for 10 min. An 80 μl aliquot of supernatant was then counted in 5 ml of Beckman Ready-SOLV EP scintillation fluid. Under these conditions, the rate of reaction was linear until over 80 % of the substrate had been hydrolysed.

UDP-galactose: *N*-acetylglucosamine galactosyltransferase assay

The UDP-galactose: *N*-acetylglucosamine galactosyltransferase assay was used as a marker of LDM membrane enrichment [18]. The protocol followed a modification of the method employed by Fleischer [19]. The assay mixture containing a 20 μl membrane suspension (20–100 μg of protein) was incubated with 20 μl of 0.2 M sodium cacodylate (pH 6.5)/3 μl of 1 M $MnCl_2$/3 μl of 1 M mercaptoethanol/5 μl of 10 % (v/v) Triton X-100/3 μl of 1 M *N*-acetylglucosamine/15 μl of 10 mM UDP-galactose containing [^{14}C]galactose (1 μCi/μmol). The assay mixture was incubated at 37 °C. It was terminated after 60 min by the addition of 17 μl of 1 M EDTA (pH 7.4). Each mixture was passed through a Dowex AG 2 × 8, 200–400 mesh column (Cl^- form) and eluted with 2 ml of distilled water. The eluate was collected in a scintillation vial and 5 ml of Beckman Ready-SOLV EP scintillation fluid added. UDP-galactose that did not undergo reaction remained bound to the column. Galactose, transferred to *N*-acetylglucosamine to form lactosamine, as well as free galactose, was eluted from the column. A control tube containing all ingredients except *N*-acetylglucosamine was added for each assay; the value obtained representing released galactose. The difference between the tubes in which *N*-acetylglucosamine is absent or present represents transferase activity. Contamination of the substrate with free radioactive sugar, or non-enzymic hydrolysis are common problems with the assay. To correct these potential problems a control (i.e. no enzyme) tube was included.

Table 1 demonstrates that there is a consistent 22 % contamination of 5′-nucleotidase activity in the LDM membrane fraction and a 24 % contamination of UDP-galactose: *N*-acetylglucosamine galatosyltransferase activity in the plasma membrane fraction. These values are within the acceptable limits for the membrane-fractionation procedure used [20–23]. Table 2 shows that the recovery of 5′-nucleotidase in the plasma membrane fraction was not altered by treatment with either insulin, glucose or glucosamine.

Determination of glucose transporter and gp160 levels

Proteins from the various membrane fractions were separated by SDS/PAGE on 10 % gels for glucose-transporter determination or 7.5 % gels for gp160 determination using the system of Laemmli [24]. The samples were electrophoresed at 500 V/40 mA for 1.5 h. Following separation, the proteins were electrophoretically transferred to PVDF membranes (500 V/50 mA for 1.5 h). The transfer buffer contained 20 mM Tris (pH 8.3)/150 mM glycine/20 % (v/v) methanol. Immunoblotting was performed using a modification of the methods described by Towbin et al. [25] and Burnette [26]. The PVDF membranes were blocked at room temperature for 2 h with 5 % skimmed milk in Tris-buffered saline (TBS) (0.9 % NaCl/10 mM Tris, pH 7.4). They were then incubated with either an anti-GLUT4 polyclonal antibody (R1159) generated to a peptide consisting of the last 12 amino acids of the C-terminal region of GLUT4 [13], an anti-GLUT1 polyclonal antibody (AP 4/93) or whole anti-gp160 serum [11] overnight at 4 °C on a rotating wheel. The PVDF membranes were then washed three times in TBS, for 10 min, and incubated with a secondary antibody, swine anti-rabbit immunoglobulins, for 1 h at room temperature. Subsequently, the PVDF membranes were washed a further five times, for 10 min in TBS, followed by a 2 h wash in TBS/Tween-20 (0.05 % Tween-20), with fresh TBS/Tween-20 added after 1 h. Following the 2 h wash, the membranes were washed twice for 10 min in TBS. Enhanced chemiluminescence was used for detection and autoradiograms were quantified by laser densitometry.

Glycosylation of gp160

Adipocytes were prepared as described above, and pre-incubated with D-[2-^{3}H]mannose (20 Ci/mmol, 40 μCi/ml) for 2 h [27] in the presence or absence of high glucose (30 mM) or glucosamine (3 mM). A total membrane fraction was prepared and solubilized as described previously [1,13] and gp160 immunoprecipitated as follows. 20 μl of Protein A–Sepharose beads and 5 μl of gp160 antibody were incubated at room temperature for 10 min. The beads were then washed with 1 ml of PBS and 1 ml of membrane lysate was added to the beads, which were incubated on a rotating wheel overnight at 4 °C. The beads were then washed three times for 1 min in a low-salt (PBS/0.2 % Triton X-100) wash solution. Following the last wash, 15 μl of PBS and 15 μl of Laemmli sample buffer were added to each tube, mixed and centrifuged at 14250 ***g*** for 1 min. Each sample was then loaded on to the gel, run and transferred as described above. Enhanced chemiluminescence was used to detect gp160 and autoradiograms were quantified by laser densitometry. The bands corresponding to gp160 were cut from the PVDF membrane and placed into scintillation vials containing 10 ml of scintillation fluid. The amount of D-[2-^{3}H]mannose radioactivity incorporated into gp160 was determined by liquid scintillation counting in a β-counter.

Protein determination

Protein concentration was determined by the Coomassie-Brilliant-Blue method described by Bradford [28] using crystalline BSA as the standard (Bio-Rad assay).

Statistical analysis

Significance was determined using the Student's *t* test for paired data.

RESULTS

Pre-incubation of rat adipocytes with high glucose (30 mM) (in the presence of 0.7 nM insulin) or glucosamine (3 mM) for only 2 h inhibited both basal and submaximal (0.7 nM) insulin-

Table 3 The effect of high glucose or glucosamine on 2-deoxyglucose transport in rat adipocytes

Adipocytes were pre-incubated in the absence and presence of high glucose (30 mM) in the presence of insulin (0.7 nM), or with glucosamine (3 mM) for a total of 2 h at 37 °C. Cells were then washed to remove extracellular insulin, glucose or glucosamine prior to further stimulation with 0.7 nM insulin for 30 min. 2-Deoxyglucose transport rates were measured in quadruplicate in four separate experiments as described in Experimental. Results are presented as fold change over basal activity in the absence of any additions. The basal transport rate was 409 ± 53 pmol/min per 10^6 cells.

	2-Deoxyglucose transport (fold increase)		
	Control	High glucose	Glucosamine
Basal	1.0 ± 0	0.71 ± 0.13*	0.46 ± 0.15*
+ Insulin	3.11 ± 0.29	2.20 ± 0.19*	1.93 ± 0.22*

* $P < 0.05$ compared with respective control.

stimulated glucose transport (Table 3 and Table 4). We have recently shown that pre-incubation of rat adipocytes with either high glucose or glucosamine results in retention of GLUT4 within the LDM compartment [1]. To investigate the mechanism by which increased flux through the hexosamine biosynthesis pathway can cause sequestration of GLUT4 intracellularly in rat adipocytes, gp160 levels were measured in LDM, HDM and plasma membranes under basal conditions following high glucose or glucosamine pre-incubation.

Both high glucose (in the presence of insulin) and glucosamine induced a decrease in basal plasma membrane GLUT4 levels by 53% and 85%, respectively ($P < 0.005$, $N = 4$) (Figure 1A, Table 5), accompanied by an increase in basal GLUT4 levels in LDM by 117% and 116%, respectively ($P < 0.005$, $N = 4$) (Figure 1B, Table 5), which confirms intracellular retention of GLUT4 as shown previously [1]. GLUT4 levels in HDM were unchanged ($P > 0.05$, not significant, $N = 4$) (Figure 1C, Table 5). In contrast, pre-incubation of adipocytes with high glucose (30 mM) or glucosamine (3 mM) decreased gp160 levels in LDM by 72% and 63%, respectively ($P < 0.0005$, $N = 4$) (Figure 2B, Table 6). The plasma membrane fraction contained no detectable levels of gp160 under any incubation condition (Figure 2A, Table 6). Given the short incubation time (2 h), this suggests that if gp160 is absent from the plasma membrane and reduced compared to control in the LDM, high glucose or glucosamine incubation may lead to increased gp160 levels in another cell compartment. Upon fractionation and isolation of HDM we observed increased levels of gp160 in this fraction under conditions of high glucose (126%, $P < 0.005$, $N = 4$) and glucosamine (109%, $P < 0.01$, $N = 4$) pre-incubation, respectively (Figure 2C, Table 6). Our observation that gp160 is present only

Table 4 The effect of high glucose or glucosamine on 3-*O*-methylglucose transport in rat adipocytes

Adipocytes were pre-incubated as described in Table 1. 3-*O*-Methylglucose transport was measured in duplicate in three separate experiments as described in Experimental. Results are presented as fold change over basal activity in the absence of any additions. The basal transport rate was 561 ± 61 pmol/min per 10^6 cells.

	3-*O*-Methylglucose transport (fold increase)		
	Control	High glucose	Glucosamine
Basal	1.0 ± 0	0.69 ± 0.11*	0.55 ± 0.18**
+ Insulin	6.07 ± 0.23	4.21 ± 0.29*	3.97 ± 0.34*

* $P < 0.05$; ** $P < 0.005$ compared with respective control.

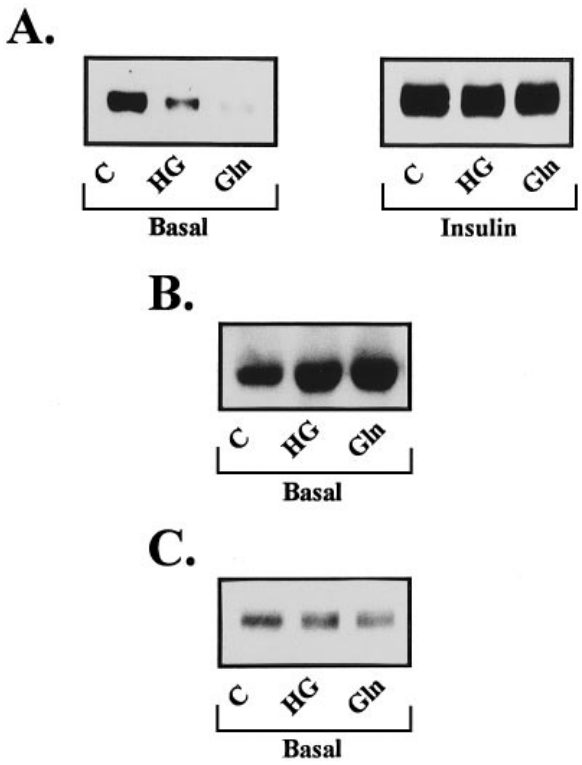

Figure 1 Representative immunoblots showing the effects of high glucose and of glucosamine on plasma membrane (A), LDM (B) and HDM (C) GLUT4 levels

Adipocytes were pre-incubated in the absence or presence of high glucose (30 mM) in the presence of insulin (0.7 nM), or with glucosamine (3 mM) for a total of 2 h at 37 °C. Cells were then washed to remove extracellular insulin, glucose or glucosamine prior to further stimulation with or without 0.7 nM insulin for 30 min. Subsequently, cells were homogenized and plasma membrane fractions obtained by differential ultracentrifugation. C, control; HG, high glucose; Gln, glucosamine.

Table 5 Effects of high glucose and of glucosamine on plasma membrane, LDM and HDM GLUT4 levels

The results from four separate experiments conducted as described in Figure 1 are presented. Autoradiographs were scanned using laser densitometry and results are means ± S.E.M. ($N = 4$). The basal control (PM, LDM, HDM) has been assigned a value of 1.

	GLUT4 levels (relative to basal control)		
Fraction	Control	High glucose	Glucosamine
Plasma membrane			
Basal	1	0.47 ± 0.08*	0.15 ± 0.05*
+ Insulin	3.88 ± 0.56	3.41 ± 0.45	3.74 ± 0.35
LDM			
Basal	1	2.17 ± 0.13*	2.16 ± 0.11*
HDM			
Basal	1	1.10 ± 0.11	0.85 ± 0.10

* $P < 0.005$ compared with control.

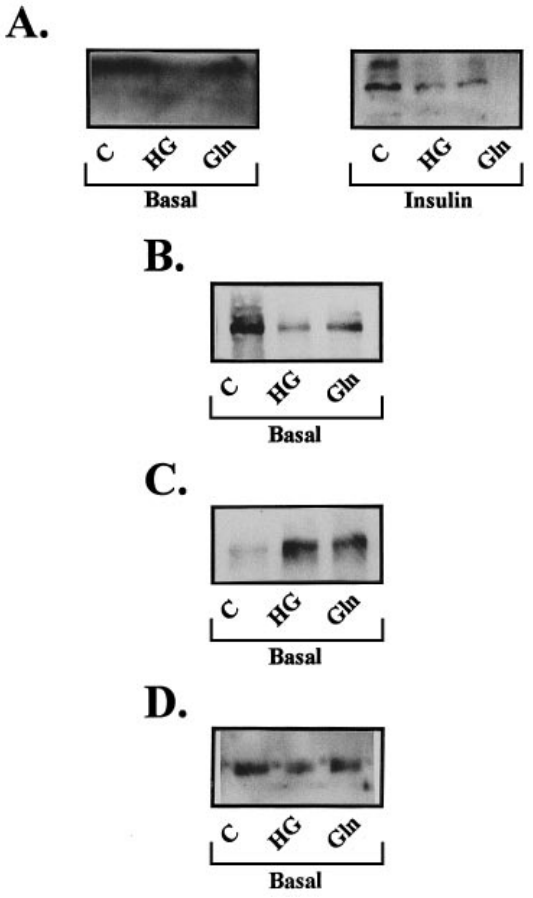

Figure 2 Representative immunoblots showing effects of high glucose and of glucosamine on plasma membrane (A), LDM (B), HDM (C) and total membrane (D) gp160 levels

Adipocytes were treated as described in the legend to Figure 1. Cells were then homogenized and plasma membrane fractions obtained by differential ultracentrifugation. C, control; HG, high glucose; Gln, glucosamine. The plasma membrane immunoblot was overexposed. This was done to show convincingly that there is no gp160 on the plasma membrane under basal conditions.

Table 6 Effects of high glucose and of glucosamine on plasma membrane, LDM, HDM and total membrane gp160 levels

Autoradiographs were scanned as described in the legend to Table 1. Results are means ± S.E.M. of four separate experiments; ND, not detectable.

	gp160 levels (relative to control)		
Fraction	Control	High glucose	Glucosamine
Plasma membrane			
Basal	ND	ND	ND
+ Insulin	1	0.41 ± 0.01†	0.36 ± 0.02†
LDM			
Basal	1	0.28 ± 0.03†	0.37 ± 0.03†
HDM			
Basal	1	2.26 ± 0.23*	2.09 ± 0.37‡
Total membrane			
Basal	1	0.92 ± 0.08	0.89 ± 0.10

† $P < 0.0005$; * $P < 0.005$; ‡ $P < 0.01$ compared with control.

Table 7 The effect of high glucose or glucosamine on the glycosylation rate of gp160 in rat adipocytes

Adipocytes were pre-incubated as described in Figure 1. D-[2-^{3}H]Mannose incorporation was measured in four separate experiments as described in Experimental. Results are presented as D-[2-^{3}H]mannose incorporation (cpm/arbitrary densitometry units of gp160). Values represent mean ± S.E.M.

	D-[2-^{3}H]Mannose incorporation (cpm/arbitrary densitometry units)		
	Control	High glucose	Glucosamine
Basal	156.74 ± 25.81	164.07 ± 16.77	181.16 ± 20.06

in LDM and HDM is in accordance with Kandror and Pilch [11], who demonstrated that in resting adipocytes gp160 is absent from the plasma membrane, cytosol and combined fraction of mitochondria and nuclei, and detectable only in the LDM and HDM [11]. In addition, we demonstrate that the various incubation conditions do not alter gp160 levels in a total membrane fraction (P = not significant, $N = 4$) (Figure 2D, Table 6), indicating that gp160 is not degraded under conditions of high glucose or glucosamine pre-incubation.

In contrast with non-stimulated cells, when adipocytes were stimulated with 0.7 nM insulin for 30 min at 37 °C, movement of GLUT4 from the LDM to the plasma membrane was unaffected by pre-incubation with high glucose or glucosamine (P = not significant, $N = 4$) (Figure 1A, Table 5). gp160 can be translocated like GLUT4 in response to insulin (Figure 2A, insulin, C); however, unlike GLUT4, it is significantly decreased in plasma membranes from insulin-stimulated cells pre-incubated with high glucose or glucosamine (59 % and 64 %, respectively; $P < 0.0005$, $N = 4$) (Figure 2A, Table 6), probably reflecting its absence from the LDM fraction under these conditions.

Incubation of adipocytes with either high glucose (in the presence of insulin) or glucosamine did not alter GLUT1 levels on the plasma membrane from either basal [(1.0 ± 0.04)-fold and (0.95 ± 0.19)-fold change over basal control values in high glucose and glucosamine conditions, respectively] or insulin-stimulated cells [(3.99 ± 0.06)-fold, (4.20 ± 0.17)-fold and (3.74 ± 0.53)-fold increases in control, high glucose and glucosamine-treated cells, respectively].

One possible explanation for the altered distribution of gp160 following high glucose or glucosamine pre-incubation is a change in the glycosylation of gp160, resulting in its retention in the HDM. Therefore, we investigated the glycosylation of gp160 by measuring the incorporation of ^{3}H-mannose, an indicator of *N*-linked and *O*-linked glycosylation. As shown in Table 7, under the present conditions, the glycosylation of gp160 was not affected by either high glucose or glucosamine following a 2 h pre-incubation (P = not significant, $N = 4$).

DISCUSSION

As shown previously, both high glucose and glucosamine decrease basal and insulin-stimulated glucose transport [1]. This was reproduced in the present study using both 2-deoxyglucose (Table 3) and, to exclude phosphorylation defects, 3-*O*-methylglucose (Table 4). The decrease in glucose transport in the insulin-stimulated state was due to a lower basal transport rate, as the fold stimulation by insulin remained the same. High glucose did not inhibit maximal (7 nM) insulin-stimulated glucose

transport [control, (2.79 ± 0.31)-fold; high glucose, (2.94 ± 0.27)-fold].

We have also previously demonstrated that pre-incubation with high glucose or glucosamine decreased basal levels of GLUT4 on the plasma membrane, with a concomitant increase in the LDM, suggesting intracellular retention of the transporter [1]. This effect appears to be a consequence of metabolism via the hexosamine-biosynthesis pathway as the effect of glucose, but not that of glucosamine, is blocked by azaserine [1]. Since GLUT4 continuously recycles in the basal state, the retention of this transporter in the LDM fraction suggests that increased flux through the hexosamine-biosynthesis pathway impairs the constitutive movement of GLUT4. We now show that, concomitant with retention of GLUT4 in the LDM, there is retention of gp160 in the HDM fraction. It is possible that the disappearance of gp160 from the LDM is linked to the intracellular retention of GLUT4, and this suggests that gp160 may be involved in constitutive GLUT4 cycling. An alternative explanation is that increased flux through the hexosamine-biosynthesis pathway retards the general movement of proteins from one compartment to another. If so, the retention of GLUT4 in the LDM and of gp160 in the HDM would not be causally linked. However, in our studies a generalized defect does not appear to explain the results, as there is no concomitant intracellular retention of GLUT1.

Kandror and Pilch [3] have shown that gp160 translocates to the cell surface in a similar fashion to GLUT4 in response to insulin; however, the role of gp160 in insulin-stimulated GLUT4 trafficking remains uncertain. Whatever its role, gp160 and GLUT4 have been co-localized under a variety of conditions. In the present study it has been possible to create, for the first time, conditions in which there is dissociation between gp160 and GLUT4. Whereas insulin normally stimulates translocation of both GLUT4 and gp160, increased flux through the hexosamine-biosynthesis pathway leads to insulin-stimulated GLUT4 translocation in the absence of gp160 translocation. The present results therefore suggest that while gp160 may play a role in constitutive GLUT4 trafficking, it does not appear to be necessary for insulin-stimulated GLUT4 trafficking.

It is known that increased activity of the hexosamine-biosynthesis pathway alters the glycosylation rate of certain proteins [29–31]. Glucosamine, has been shown to inhibit N-linked glycosylation [29], possibly by causing an increase in UDP-*N*-acetylglucosamine and a concomitant decrease in UTP, UDP, UMP, UDP-glucose and UDP-galactose [32]. UDP-glucose and other UDP-linked sugars are obligatory intermediates for the processing of glycoproteins. Glucosamine interferes with insulin receptor processing causing accumulation of insulin receptors in the Golgi apparatus with a reduction in membrane-associated receptors [30,33]. Mutation of the N-terminal glycosylation sites of the insulin receptor causes a similar processing defect [34]. In contrast, Hawkins et al. [35], using ^{14}C-labelled glucosamine injected into rats have reported increased overall labelling of proteins in GLUT4-containing vesicles during infusion of 30 μmol of glucosamine/kg per min. It is possible therefore that high glucose or glucosamine, by increasing flux through the hexosamine-biosynthesis pathway, interfere with processing of proteins whose glycosylation is necessary for vesicular trafficking. Thus, alteration of glycosylation of proteins on GLUT4-containing vesicles, such as gp160, may explain the retention of GLUT4 in the intracellular compartment (LDM). In the present study we could not demonstrate a change in the glycosylation of gp160 using D-[2-^{3}H]mannose; however, a change in its glycosylation state cannot be excluded as the choice of labelled sugar may be critical.

The effects reported in the present study were obtained with a pre-incubation period of only 2 h in high glucose or glucosamine. It is likely that additional effects will be obtained with longer pre-incubation times. Previous studies involving longer periods of exposure to hyperglycaemia [36] or glucosamine [37] have reported defects in insulin-stimulated GLUT4 translocation. The profound decrease in gp160 in LDM after only 2 h pre-incubation suggests that this protein may have a rapid turnover. One could hypothesize that longer pre-incubation times may cause retention of other GLUT4-vesicle-associated proteins that have a slower turnover rate. Some of these may be involved in insulin-stimulated GLUT4 translocation.

In conclusion, we present evidence that gp160 is not required for insulin-mediated translocation of GLUT4, but may be involved in constitutive GLUT4 cycling.

This work was supported by a program grant from the NH and MRC of Australia and from a grant-in-aid from Diabetes Australia. We would like to thank Professor D. James, Centre for Molecular and Cellular Biology, University of Queensland, St. Lucia, Queensland, Australia for supplying the GLUT1 antibody and Professor P. Pilch and Associate Professor K. Kandror, Department of Biochemistry, School of Medicine, Boston University, Boston, MA, U.S.A. for generously supplying the gp160 antibody.

REFERENCES

1 Filippis, A., Clark, S. and Proietto, J. (1997) Biochem. J. **324**, 981–985
2 Jhun, B. H., Rampal, A. L., Liu. H., Lachaal, M. and Jung, C. Y. (1992) J. Biol. Chem. **267**, 17710–17715
3 Kandror, K. V. and Pilch, P. F. (1994) J. Biol. Chem. **269**, 138–142
4 Thoidis, G., Kotliar, N. and Pilch, P. F. (1993) J. Biol. Chem. **268**, 11691–11696
5 Laurie, S. M., Cain, C. C., Lienhard, G. E. and Castle, J. D. (1993) J. Biol. Chem. **268**, 19110–19117
6 Cain, C. C., Trimble, W. S. and Lienhard, G. E. (1992) J. Biol. Chem. **267**, 11681–11684
7 Del Vecchio, R. L. and Pilch, P. F. (1991) J. Biol. Chem. **266**, 13278–13283
8 Cormont, M., Tanti, J.-F., Grémeaux, T., Van Obberghen, E. and Le Marchand-Brustel, Y. (1991) Endocrinology **129**, 3343–3350
9 Stephens, J. M. and Pilch, P. F. (1995) Endocr. Rev. **16**, 529–546
10 Mastick, C. C., Aebersold, R. and Lienhard, G. E. (1994) J. Biol. Chem. **269**, 6089–6092
11 Kandror, K. V. and Pilch, P. F. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 8017–8021
12 Rodbell, M. (1964) J. Biol. Chem. **239**, 375–380
13 Kelada, A. S. M., Macaulay, S. L. and Proietto, J. (1992) J. Biol. Chem. **267**, 7021–7025
14 Häring, H. U., Biermann, E. and Kemmler, W. (1981) Am. J. Physiol. **240**, E556–E565
15 Traxinger, R. R. and Marshall, S. (1990) Biochem. Int. **22**, 607–615
16 Stanley, K. K. and Luzio, J. P. (1979) Biochem. Soc. Trans. **7**, 361–362
17 Avruch, J. and Wallach, D. F. H. (1971) Biochim. Biophys. Acta **233**, 344–347
18 Suzuki, K. and Kono, T. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 2542–2545
19 Fleischer, B. (1974) Methods Enzymol. **29**, 180–191
20 Kono, T., Suzuki, K., Dansey, L. E., Robinson, F. W. and Blevins, T. L. (1981) J. Biol. Chem. **256**, 6400–6407
21 Simpson, I. A., Yver, D. R., Hissin, P. J., Wardzala, L. J., Karnieli, E., Salans, L. B. and Cushman, S. W. (1983) Biochim. Biophys. Acta **763**, 393–407
22 Smith, M. M., Robinson, F. W., Watanabe, T. and Kono, T. (1984) Biochim. Biophys. Acta **775**, 121–128
23 Guerre-Millo, M., Lavau, M., Horne, J. S. and Wardzala, L. J. (1985) J. Biol. Chem. **260**, 2197–2201
24 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
25 Towbin, H., Ftaehlin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
26 Burnette, W. N. (1981) Anal. Biochem. **112**, 195–203
27 Deruisseau, S., Franc, J. L., Gruffat, D. and Chabaud, O. (1994) Endocrinology **134**, 1676–1684
28 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
29 Elbein, A. D. (1987) Annu. Rev. Biochem. **56**, 497–534
30 Robinson, K. A., Sens, D. A. and Buse, M. G. (1993) Diabetes **42**, 1333–1346
31 Robinson, K. A., Weinstein, M. L., Lindenmayer, G. E. and Buse, M. G. (1995) Diabetes **44**, 1438–1446

32 Keppler, O. R., Rudigier, J. F. M., Bischoff, E. and Decker, K. F. A. (1970) Eur. J. Biochem. **17**, 246–253
33 Taylor, S. I., Kadowaki, T., Kadowaki, H., Accili, D., Cama, A. and McKeon, C. (1990) Diabetes Care **13**, 257–279
34 Caro, L. H. P., Ohali, A., Gorden, P. and Collier, E. (1994) Diabetes **43**, 240–246
35 Hawkins, M., Angelov, I., Liu, R., Brazalai, N. and Rossetti, L. (1997) J. Biol. Chem. **272**, 4889–4895
36 Napoli, R., Hirshman, M. F. and Horton, E. S. (1995) J. Clin. Invest. **96**, 427–437
37 Baron, A. D., Zhu, J. S., Weldon, H., Mainanu, L. and Garvey, W. T. (1995) J. Clin. Invest. **96**, 2792–2801

Received 8 August 1997/31 October 1997; accepted 11 November 1997