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Two glucose transporter isoforms are sorted differentially and are expressed in distinct cellular compartments

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Rat GLUT4 (adipocyte/muscle-type glucose transporter) was expressed in two fibroblastic cell lines, Chinese hamster ovary (CHO) cells and 3T3-L1 fibroblasts, under the control of the methallothionein I promoter. Although immunoblotting with a GLUT4-specific anti-peptide antibody demonstrated that the amount of GLUT4 expressed was comparable with that in 3T3-L1 adipocytes and rat adipose tissues, no increase in 2-deoxy-D-glucose uptake was observed in the basal state in fibroblasts. Immunocytochemical studies showed that the expressed GLUT4 appeared to be localized in a specific region in the cytoplasm. These results were in marked contrast to those obtained in CHO cells expressing GLUT1 (HepG2/erythrocyte-type glucose transporter) using the same expression vector. In this case the expressed GLUT1 protein appeared to reside mainly on the plasma membranes, and a significant increase in glucose uptake was observed. Although insulin increased glucose uptake in CHO cells and 3T3-L1 fibroblasts as well as in the cells expressing rat GLUT4, an increment due to insulin above basal values was small, at most 2-fold, and no significant differences were observed in insulin-stimulated glucose uptake between transfected and parental cells. In addition, no apparent differences in the subcellular distribution of expressed GLUT4 were observed between the insulin-stimulated and the basal state. These results indicate that in fibroblastic cell lines GLUT1 and GLUT4 proteins are sorted in a different fashion, and the expression of GLUT4 protein *per se* is not enough to produce a large insulin-induced increase in glucose transport activity such as that observed in rat adipocytes and 3T3-L1 adipocytes. Thus unidentified aspects of the cellular environment which are present in the adipocytes but not in fibroblastic cell lines may be required for a large insulin-induced increase in glucose transport activity to be observed.

INTRODUCTION

Recent cDNA cloning studies have shown the existence of a family of facilitative glucose transporters. This family includes GLUT1 (HepG2/erythrocyte-type), GLUT2 (liver-type) and GLUT4 (muscle/adipocyte-type) transporters, as well as others [1–12]. Although these isoforms are predicted to have very similar structures involving 12 membrane-spanning helices, each isoform has a specific tissue distribution, and GLUT4 is exclusively expressed in fat and muscle cells, which are considered to be insulin-responsive tissues [5–10]. Although insulin also increases glucose transport activity in fibroblasts, which express GLUT1, the increment induced by insulin is not greater than 2-fold (i.e. a small response). This is in marked contrast to the insulin effect on glucose transport activity in fat cells, in which the response is usually more than 10-fold (i.e. a large response). These results have raised a possibility that the presence of GLUT4 contributes to the large response which is observed in insulin-responsive tissues. To address this issue, rat GLUT4 was expressed in two fibroblastic cell lines, Chinese hamster ovary (CHO) cells and 3T3-L1 fibroblasts, and the glucose transport activity was examined. We also studied the subcellular distribution of GLUT1 and GLUT4 proteins which were expressed by using the same expression vector. Here we report that GLUT4 was expressed in a subcellular region distinct from that for expressed GLUT1, and that the expressed GLUT4 did not appear to contribute to glucose transport activity in fibroblasts in the absence and even in the presence of insulin.

EXPERIMENTAL

Expression of rat GLUT4 and Western blotting

A full-length rat GLUT4 cDNA was cloned from a rat adipose tissue cDNA library (Clonetech, San Francisco, CA, U.S.A.) by using oligonucleotide probes of nucleotides 149–175 and 1662–1691 of the rat GLUT4 nucleotide sequence [5]. The first screening of the library was carried out with the probe of nucleotides 1662–1691. The oligonucleotide was labelled with T4 polynucleotide kinase, and hybridization was performed in 40% formamide, 5 × Denhardt's solution (0.1% each of BSA, polyvinylpyrrolidone and Ficoll), 5 × SSPE (0.75 M-NaCl/0.05 M-NaH_2PO_4/5 mM-EDTA, pH 7.4), 0.5% SDS and 100 μg of *Escherichia coli* tRNA/ml at 37 °C for 18 h [13]. The filters were washed with 2 × SCC (0.3 M-NaCl/0.03 M-sodium citrate) for 30 min at 37 °C and then autoradiographed. The positive clones were screened with another oligonucleotide probe, of nucleotides 149–175. Hybridization and washing were carried out using the same procedures as for the first screening. Since the coding sequence of rat GLUT4 cDNA [5] is nucleotides 154–1680, the clones positive with both oligonucleotide probes were expected to be full-length GLUT4 cDNAs. In fact, the positive clones were plaque-purified and the whole inserts were verified to be full-length GLUT4 cDNAs by sequencing by the dideoxy sequence method [13]. A *DraI–XbaI* 1.8 b fragment containing the whole coding sequence was excised and subcloned into the pMTH-neo vector [14,15], yielding pMTH-GLUT4. CHO cells were maintained in Ham F-12 medium containing 10% fetal

Abbreviations used: CHO, Chinese hamster ovary; PMSF, phenylmethanesulphonyl fluoride; PBS, phosphate-buffered saline; FCS, fetal calf serum; GLUT4, adipocyte/muscle-type glucose transporter; GLUT1, rat brain/HepG2/erythrocyte-type glucose transporter; FITC, fluorescein isothiocyanate; ATB-BMPA, 2-*N*-[4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis-(D-mannos-4-yloxy)]-2-propylamine.

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calf serum (FCS) in 5% CO_2. 3T3-L1 fibroblasts were maintained in Dulbecco's modified Eagle's medium containing 10% FCS in 10% CO_2.

Transfections were carried out with pMTH-neo or pMTH-GLUT4 by the calcium phosphate method as described previously [15]. G418 (neomycin derivative)-resistant clones were isolated and screened for GLUT4 expression.

An antibody was prepared against the synthetic peptide of the *C*-terminal domain (TPEELFHPLGADSQV) of GLUT1, and was designated αGLUT1-C in this study. The preparation and characterization of this antibody was described previously [16]. An antibody against the synthetic peptide of the *C*-terminal domain (KPSTELEYLGPDEND) of rat GLUT4 was prepared using the same procedures as for α-GLUT1-C, and was designated αGLUT4-C in this study. αGLUT4-C did not react with GLUT1 in human erythrocytes or with GLUT2 in rat liver (results not shown). However, αGLUT4-C recognized a protein with an apparent molecular mass of 50 kDa in rat adipocytes [17], and the amount of this protein was decreased in the low-density microsomes prepared from insulin-treated adipocytes compared with those from control adipocytes (results not shown). These results indicate that αGLUT4-C specifically recognizes rat GLUT4. Affinity-purified antibodies were prepared using the short peptide affinity column as described previously [16]. Western blot analysis was performed as described previously [16] using affinity-purified αGLUT1-C or αGLUT4-C antibody. Cells were homogenized in 10 mM-Tris, 1 mM-EDTA, 1 mM-phenylmethanesulphonyl fluoride (PMSF) and 250 mM-sucrose, pH 7.4, in a Potter–Elvehjem glass–Teflon type homogenizer at 4 °C. The homogenates were centrifuged at 900 *g* for 10 min to sediment the fraction containing mainly nuclei, and the resulting supernatant was centrifuged at 170000 *g* for 75 min at 4 °C. The pellet (100 μg) was subjected to SDS/polyacrylamide (10%)-gel electrophoresis, transferred to nitrocellulose paper and incubated with 5 μg of the affinity-purified αGLUT4-C/ml, followed by ^{125}I-labelled Protein A (Amersham). Several clones with high levels of expression were selected for further experiments.

Measurement of glucose uptake

Glucose uptake activity in CHO cells and 3T3-L1 cells was determined by measuring the uptake of 2-deoxy-D-glucose as previously described [15], with some modifications. Near-confluent monolayers of CHO cells in 24-well plates and of 3T3-L1 cells in 12-well plates were incubated with 0.1 mM-2-deoxy-D-[1,2-^{3}H]glucose (ICN) for 5 min at 37 °C. The reaction was terminated by the addition of ice-cold Krebs–Ringer phosphate buffer containing 0.3 mM-phloretin. The cells were washed with ice-cold 10 mM-phosphate-buffered saline (PBS; 138 mM-NaCl, 2.7 mM-KCl, 8.1 mM-Na_2HPO_4 and 1.8 mM-KH_2PO_4) containing 10 mM-glucose and solubilized with 0.05% SDS, and the radioactivity was measured.

Cell surface labelling of the glucose transporter

Labelling of the cell surface glycoproteins was performed according to the methods described previously [18], with some modifications [19]. The cells in the culture dish (60 mm diameter) were incubated with 1 unit of neuraminidase/ml (Sigma) at 22 °C in 20 mM-PBS, pH 6.0, for 15 min. The procedures for labelling were performed at 15 °C. The cells were pre-reduced by the addition of 8 μl of 250 mM-$NaBH_4$ in 100 mM-NaOH to 1 ml of 20 mM-PBS, pH 8.0. After 5 min the cells were washed and incubated with 40 units of galactose oxidase (Sigma) in 1 ml of 20 mM-PBS, pH 7.4, for 30 min. The cells were then reduced by the addition of the indicated amount of NaB^3H_4 (Dupont–New England Nuclear) in 100 mM-NaOH to 1 ml of 20 mM-PBS. pH 8.0. After 10 min, 1 ml of 20 mM-PBS, pH 7.4, was added and the cells were further incubated for 5 min. The cells were then washed and solubilized with 1 ml of 2% dodecyl octaethyleneglycol ether in 50 mM-Hepes/150 mM-NaCl, pH 7.6, containing 1 mM-PMSF. The cell lysate was centrifuged at 13000 *g* for 30 min at 4 °C. Immunoprecipitation of the labelled glucose transporter in the supernatant was performed with 30 μg of affinity-purified αGLUT1-C or αGLUT4-C and 25 μl of Protein A–Cellulofine (Seikagaku Kogyo, Tokyo, Japan). The immunoprecipitates were suspended in 2% SDS/20 mM-dithiothreitol/6 M-urea and then subjected to SDS/polyacrylamide (10%)-gel electrophoresis. The gel lanes were sliced and the incorporated ^{3}H in each gel slice (5 mm long) was determined as previously described [16].

Immunofluorescence labelling of glucose transporters

The control CHO cells and the transfected CHO cells were grown in chamber slides (Lab-Tek, Nunc) and treated with absolute methanol for 2 min at 24 °C. The cells were incubated with PBS-C (10 mM-PBS supplemented with 0.9 mM-$CaCl_2$ and 0.9 mM-$MgSO_4$) containing 0.1% Triton X-100 for 5 min, and subsequently incubated with 5 μg of affinity-purified α-GLUT4-C or α-GLUT1-C/ml in PBS-C for 45 min at 24 °C. The cells were washed with 3 × 1 ml of PBS-C and further incubated with 30 μg of fluorescein isothiocyanate (FITC)-conjugated goat anti-(rabbit IgG) (Funakoshi, Tokyo, Japan) for 30 min at 24 °C. The cells were washed three times with PBS-C and the slides were viewed on a fluorescence microscope using the appropriate filters.

RESULTS

Stable expression of rat GLUT4 in CHO and 3T3-L1 cell lines is shown in Fig. 1. Western blot analysis of the cell membranes using the anti-peptide antibody αGLUT4-C demonstrated that a large amount of the protein of apparent molecular mass 50 kDa was expressed in transfected CHO cells (Fig. 1*a*, lanes 7 and 8) and 3T3-L1 cells (Fig. 1*b*, lanes 4 and 5). This protein band was not observed in control CHO cells (Fig. 1*a*, lane 6) or control 3T3-L1 cells (Fig. 1*b*, lane 3). In addition, this band also reacted with the anti-peptide antibody against the *N*-terminal or middle cytoplasmic loop region of rat GLUT4, but not with control rabbit IgG (results not shown). Several cell clones (CHO-GLUT4 A1–8 and C2–5, and 3T3-GLUT4 15 and 49) and the cells transfected with vector alone were used for further experiments. The amount of GLUT4 protein expressed in these cells was estimated by comparison with the amount of GLUT4 in the total membranes from rat adipose tissue or in the low-density microsomes from isolated rat adipocytes. The amount of expressed GLUT4 in the total membranes of CHO cells (Fig. 1*a*, lanes 7 and 8) was almost comparable with the amount of rat GLUT4 in total membranes of rat epididymal fat tissues (Fig. 1*a*, lane 5). It should be noted that the expressed GLUT4 had a slightly lower electrophoretic mobility than rat adipocyte GLUT4.

We have developed a peptide-based radioimmunoassay for the glucose transporter isoform GLUT1 [20]. By using the same strategy we have also developed peptide-based radioimmunoassay systems specific for other isoforms, including GLUT4 (K. Tsukuda & Y. Oka, unpublished work). The amount of GLUT4 protein was estimated by this radioimmunoassay system specific for GLUT4 in the sample used for the experiment shown in Fig. 1. The total membranes of rat epididymal fat tissues, clones CHO-GLUT4 A1–8 and CHO-GLUT4 C2–5, and the low-density microsomes of rat adipocytes were estimated to contain 7.3, 4.2, 5.5 and 28.5 pmol of GLUT4/mg of membrane respectively. The validity of this radioimmunoassay was supported by the fact that a good correlation was observed between the relative abundance of GLUT4, calculated from these values,

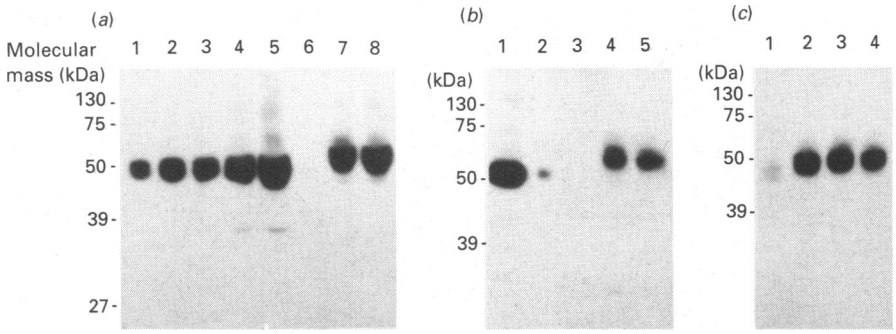

Fig. 1. Western blotting analysis of control and transfected cell lines

(*a*) Expressed GLUT4 protein in CHO-GLUT4 cells compared with GLUT4 in rat adipose tissue. Prepared membranes were subjected to SDS/polyacrylamide (10%)-gel electrophoresis, transferred to a nitrocellulose membrane and incubated with affinity-purified αGLUT4-C followed with ^{125}I-Protein A. Membranes: low-density microsomes of rat adipocytes (5 μg in lane 1 and 10 μg in lane 2), and total membranes of rat adipose tissue (25, 50 and 100 μg in lanes 3, 4 and 5 respectively), control CHO cells (100 μg, lane 6), CHO-GLUT4 A1–8 cells (100 μg, lane 7) and CHO-GLUT4 C2–5 cells (100 μg, lane 8). (*b*) Expressed GLUT4 in protein in 3T3-GLUT4 cells compared with GLUT4 in rat adipose tissue. Total membranes (100 μg) were from adipose tissue (lane 1), control 3T3-L1 fibroblasts (lane 3), 3T3-GLUT4 15 cells (lane 4) and 3T3-GLUT4 49 cells (lane 5). Lane 2 contains BSA (10 μg). (*c*) Endogenous GLUT1 protein. Western blotting of total membrane (100 μg) was performed with affinity-purified αGLUT1-C. Samples were rat epididymal fat tissue (lane 1), control 3T3-L1 fibroblasts (lane 2), 3T3-GLUT4 15 cells (lane 3) and 3T3-GLUT4 49 cells (lane 4).

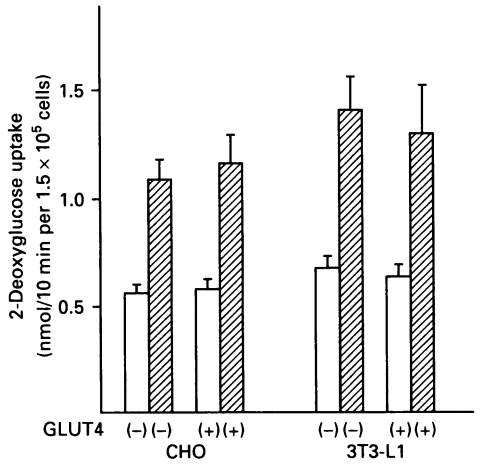

Fig. 2. 2-Deoxyglucose uptake in control and transfected cell lines

2-Deoxy-[^{3}H]glucose uptake measurements were performed in control (□) and insulin (0.1 μM)-treated (▨) cells as described previously [15]. Near-confluent monolayers of cells were incubated with 0.1 mM-2-deoxy-D-[1,2-^{3}H]glucose for 5 min at 37 °C. The reaction was stopped by adding ice-cold PBS containing 10 mM-glucose and 0.3 mM-phloretin, followed by washing three times with ice-cold PBS containing 10 mM-glucose. The cells were subsequently solubilized with 0.05% SDS and the radioactivity was measured. The values were means ± S.D. of three experiments. CHO GLUT4(−), control CHO cell line; CHO GLUT4(+), CHO-GLUT4 A1–8 cells; 3T3-L1 GLUT4(−), control 3T3-L1 fibroblasts; 3T3-L1 GLUT4(+), 3T3-GLUT4 49 cells.

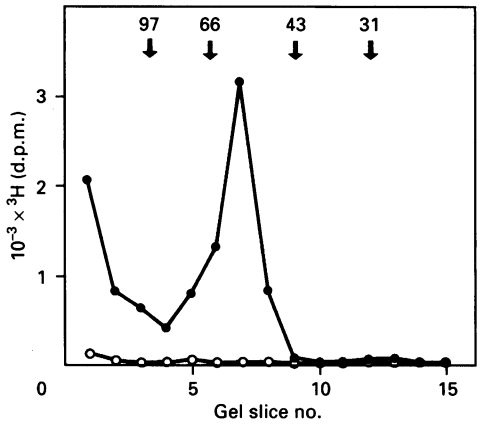

Fig. 3. Labelling of cell surface glucose transporters

Cell surface glycoproteins of CHO-GLUT4 A1–8 cells (○) and CHO-GLUT1 cells (●) were labelled with galactose oxidase and 18.5 MBq of NaB^3H_4 in intact cells and subsequently immunoprecipitated with αGLUT4-C (CHO-GLUT4 A1–8 cells) or with α-GLUT-C (CHO-GLUT1 cells). The pellets were suspended in 1% SDS/50 mM-dithiothreitol and electrophoresed on a 10% polyacrylamide gel. The gel lanes were sliced and the radioactivity was determined. As the immunoprecipitate from the control cells with αGLUT4-C showed similar results to that from CHO-GLUT4 cells with αGLUT4-C, the former results were omitted for the sake of clarity. The experiments were performed three times with similar results. Values at the top of the Figure indicate molecular mass (kDa).

and the relative density of the GLUT4 band on Western blotting (Fig. 1*a*). Furthermore, the estimated concentration of glucose transporter in the rat adipocyte low-density microsomes was slightly lower than but in the range of the previous result, i.e. approx. 42 pmol/mg of membrane, based on cytochalasin B binding (the value is estimated from the Fig. 3 in ref. [21]). Expression of the exogenous GLUT4 protein did not affect the expression of the endogenous GLUT1 protein in 3T3-L1 fibroblasts (Fig. 1*c*). Western blotting with the anti-peptide antibody αGLUT1-C demonstrated that control 3T3-L1 fibroblasts (Fig. 1*c*, lane 2) and 3T3-GLUT4 cells (lanes 3 and 4) contained similar amounts of endogenous GLUT1, whereas adipose tissue contained relatively low levels of GLUT1 protein (Fig. 1*c*, lane 1).

Interestingly, 2-deoxy-D-glucose uptake was not increased in CHO-GLUT4 cells compared with control CHO cells (Fig. 2), although the amount of expressed GLUT4 appeared to be sufficient to induce a significant increase in glucose transport activity if it were expressed on the cell surface. By employing the peptide-based radioimmunoassay specific for GLUT1 [20], one of the CHO clones expressing rabbit GLUT1 was observed to contain 6.1 pmol of GLUT1/mg of total cell membranes.

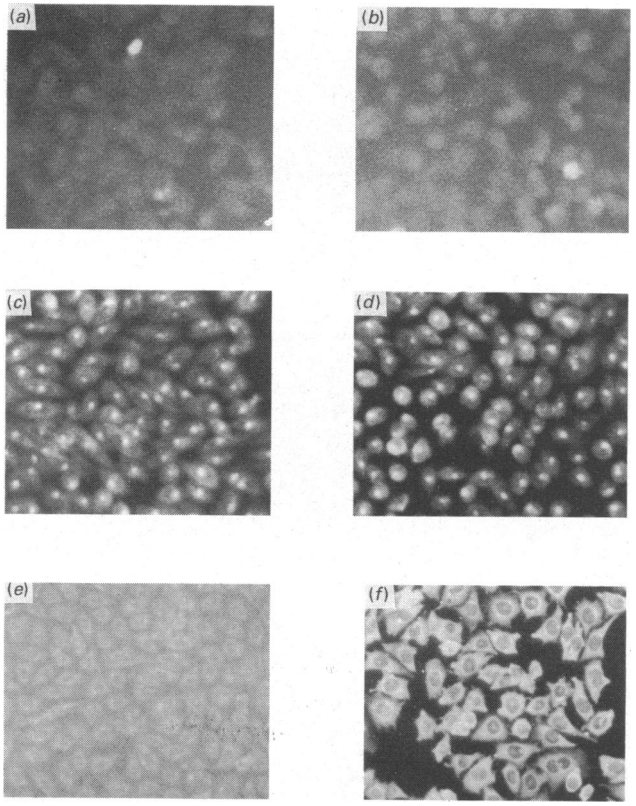

Fig. 4. Immunocytochemical staining of glucose transporters

Control CHO cells and GLUT1- or GLUT4-transfected CHO cells were grown on chamber slides. After treatment with methanol and Triton X-100, the cells were incubated with affinity-purified αGLUT4-C or αGLUT1-C at a concentration of 5 μg/ml for 30 min at 24 °C. After washing with PBS-C, cells were stained with FITC-conjugated goat anti-(rabbit IgG) for 30 min. (*a*) CHO-GLUT4 C2–5 cells incubated with control IgG; (*b*) control CHO cells incubated with αGLUT4-C; (*c*) CHO-GLUT4 C2–5 cells incubated with αGLUT4-C in the absence of insulin; (*d*) CHO-GLUT4 C2–5 cells pretreated with insulin and then incubated with αGLUT4-C; (*e*) control CHO cells with αGLUT1-C; (*f*) CHO cells expressing a large amount of rabbit GLUT1 (clone B in ref. [15]) with αGLUT1-C.

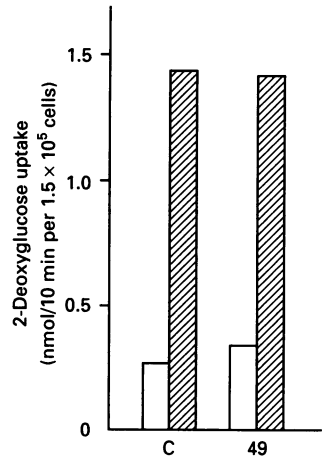

Fig. 5. Glucose transport activity in 3T3-L1 cells after differentiation

Control 3T3-L1 cells (C) and transfected 3T3-GLUT4 49 cells (49) were differentiated into adipocytes as described [23]. Glucose uptake was measured as described in the legend to Fig. 2. □, 2-Deoxyglucose uptake in the absence of insulin; ▨, 2-deoxyglucose uptake in the presence of insulin (0.1 μM). The experiments were performed three times for control 3T3-L1 cells and twice for 3T3-GLUT4 49 cells, and mean values are presented.

2-Deoxy-D-glucose uptake in this CHO clone was increased 2.5-fold compared with control CHO cells, whereas CHO-GLUT4 A1–8 cells, containing 4.2 pmol of GLUT4/mg of membranes, showed no increase in 2-deoxy-D-glucose uptake. It is difficult to explain why no increase in transport activity was observed in GLUT4 A1–8 cells if we assume that the intrinsic activity of GLUT4 is not so much decreased compared with that of GLUT1 and that GLUT4 protein is expressed in the same fashion as GLUT1 protein in CHO cells. Results similar to those observed in CHO-GLUT4 cells were obtained in 3T3-GLUT4 49 cells (Fig. 2). This cell line was estimated to contain 2.6 pmol of GLUT4/mg of total membrane, based on the density of the glucose transporter band (Fig. 1*b*, lane 5) compared with that in adipose tissue (Fig. 1*b*, lane 1).

Since GLUT4 has been shown to reside mainly in the cell interior of rat adipocytes in the basal state [10], it is possible that GLUT4 might move to the plasma membrane only after stimulation by insulin and then function as a glucose carrier for the cells. Therefore glucose uptake was also studied in the presence of insulin. Although glucose uptake was increased in both control and transfected cells, no significant differences in insulin-stimulated glucose transport activity were observed between the control and the transfected cells (CHO-GLUT4, 3T3-GLUT4). The stimulation of glucose transport activity by insulin was by up to 2-fold in the transfected cells, a similar percentage increase to that observed in control cells (Fig. 2). Although it is possible that the DNA construct is not appropriate, we obtained similar results with another construct (*Dra*I–*Hin*cII fragment of GLUT4 nucleotides 143–2490) (results not shown).

To study the reason for the apparent loss of function of expressed GLUT4 protein, the amount of cell surface glucose transporter was determined. Cell surface glycoproteins were labelled with galactose oxidase and NaB^3H_4, immunoprecipitated with αGLUT4-C and analysed on SDS/polyacrylamide gels. As shown in Fig. 3, there was no significant increase in the amount of labelled glucose transporter protein in cells expressing GLUT4 (Fig. 3) compared with control cells (results not shown). These results are quite different from those in CHO cells expressing GLUT1 (CHO-GLUT1) (Fig. 3). In addition, no significant increase in the amount of labelled GLUT4 protein was observed in CHO-GLUT4 cells in the presence of insulin (results not shown). To further confirm these results, affinity labelling of cell surface glucose transporter with 2-*N*-[4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis-(D-mannos-4-yloxy)]-2-propylamine (ATB-BMPA; generously provided by Dr. G. D. Holman, University of Bath, Bath, U.K.) [22] was carried out. It has been shown that this reagent does not enter the cells, and binds to glucose transporter on the outside of the cells. Again, no significant difference in the amount of labelled glucose transporter was observed between control and GLUT4-transfected cells (results not shown), while a large increase in the labelling of glucose transporter was observed in CHO-GLUT1 cells [19].

To further investigate the subcellular localization of the expressed GLUT4 protein in CHO-GLUT4 cells, cells were stained with αGLUT4-C and FITC-conjugated anti-(rabbit IgG). While no specific staining was observed in CHO-GLUT4 incubated with control IgG (Fig. 4*a*) or in control CHO cells incubated with αGLUT4-C (Fig. 4*b*), GLUT4 protein was observed in a specific cytoplasmic region of CHO-GLUT4 cells when incubated with αGLUT4-C (Fig. 4*c*). This staining pattern is completely different from that in CHO-GLUT1 cells incubated with αGLUT1-C (Fig. 4*f*), in which the fluorescence was observed all over the cells. Interestingly, the subcellular localization of exogenous GLUT4 was not altered by insulin treatment of CHO-

GLUT4 cells (Figs. 4*c* and 4*d*). Taken together with the results obtained with surface labelling of the glucose transporter, these results suggest that the expressed GLUT4 does not reach the plasma membrane even in the presence of insulin. Thus, the large response of glucose transport activity induced by insulin which is observed in rat adipocytes and 3T3-L1 adipocytes seems to require other conditions which do not exist in fibroblasts.

To investigate whether or not the conditions present in differentiated adipocytes are required for the insulin-responsiveness of GLUT4, control and transfected 3T3-L1 cell lines were differentiated into adipocytes using standard procedures [23] (Fig. 5). Insulin stimulated glucose transport activity by approx. 5-fold above the basal value in control 3T3-L1 adipocytes, i.e. a large response was observed. This is mostly due to a decrease in basal transport activity after differentiation in 3T3-L1 cells (compare the open bar in Fig. 5 with the open bar in Fig. 2). However, expression of GLUT4 did not induce an apparent change in glucose transport activity in 3T3-L1 adipocytes, similar to the observation in CHO-GLUT4 cells and 3T3-GLUT4 fibroblasts; no significant increase in glucose transport activity was observed between control 3T3-L1 adipocytes and 3T3-GLUT4 adipocytes not only in the absence but also in the presence of insulin.

DISCUSSION

Two glucose transporter isoforms, GLUT4 and GLUT1, showed strikingly different characteristics in their expression in CHO cells and 3T3-L1 fibroblasts. These two isoforms were expressed using the same promoter and in the same cell lines. However, GLUT1 appears to reside mainly in the plasma membranes (Fig. 4*f*), whereas GLUT4 appears not to be present in the plasma membranes but rather to be localized in an undefined intracellular compartment (Fig. 4*c*). No significant integration of the expressed GLUT4 into the cell surface membrane was demonstrated by labelling of glucose transporters on the cell surface using galactose oxidase and NaB^3H_4 (Fig. 3) or ATB-BMPA. Consistent with the concept of a subcellular localization of the expressed GLUT4 protein (Fig. 4*c*), no significant increase in glucose transport activity was observed in transfected compared with non-transfected cells in the basal state (Fig. 2). Since GLUT4 and GLUT1 are very likely to go via a common route in the early stage of biosynthesis, a sorting location which clearly distinguishes these similar isoforms (e.g. trans Golgi network) must exist. Also, there must be a sorting signal in the specific regions of each type of glucose transporter molecule.

It has been reported that acquisition of a large response to insulin in glucose transport activity correlates with the appearance of GLUT4 protein in 3T3-L1 adipocytes [24]. Therefore, the lack of a large response to insulin in fibroblasts has been suggested to result from the absence of GLUT4 protein [24]. We previously observed that the expressed GLUT1 responded to insulin, but that the responsiveness remained as small as that with endogenous GLUT1 [15]. However, even when GLUT4 was expressed in two fibroblastic cell lines, insulin-stimulated glucose uptake did not increase by more than 2-fold (i.e. a small response). Thus the present observation clearly indicates that the expression of GLUT4 *per se* is not enough for a high degree of insulin responsiveness in glucose transport activity. The amount of GLUT1 is not different between control and transfected fibroblasts, and insulin is likely to exert a small stimulatory effect on glucose transport activity in the transfected cells via actions on GLUT1 but not on GLUT4. This is further supported by immunocytochemical studies showing no apparent alterations by insulin in the subcellular localization of GLUT4 (Figs. 4*c* and 4*d*). Also, cell surface labelling of GLUT4 indicated no significant increase in GLUT4 on the cell surface on exposure to insulin. Thus the expressed GLUT4 does not seem to contribute to transport activity in the absence and even in the presence of insulin. This is again quite different from the results obtained in CHO-GLUT1 cells; expressed GLUT1 contributes to glucose transport activity in the basal state, and also responds to insulin [15].

One might argue that the overexpressed foreign protein, GLUT4, might be incorrectly folded and thus fail to migrate beyond the endoplasmic reticulum or the cis stacks of the Golgi. Although incorrect folding is possible, it is very unlikely that our observations resulted from overexpression of GLUT4, since the level of expression of this protein in our transfected cells is similar to that in rat epididymal adipocytes and in 3T3-L1 adipocytes (Figs. 1*a* and 1*b*). In addition, the amount of expressed GLUT4 in CHO-GLUT4 cells is much less than the amount of expressed GLUT1 in our transfected CHO-GLUT1 cells. The highest expression level of GLUT1 in the CHO-GLUT1 cell lines is nearly 100 pmol/mg of membrane [20]. It should be noted that GLUT1 was expressed using the same expression vector as was used for GLUT4 expression, and that the expressed GLUT1 in CHO cells appeared to be properly folded and sorted, as indicated in Fig. 4(*f*), despite its high expression level. Proper folding and sorting of the expressed GLUT1 is also indicated by the increase in glucose transport activity induced by expression of GLUT1 [15,19].

Incorrect folding of the expressed GLUT4 might be suggested by the fact that it exhibited a lower electrophoretic mobility than the GLUT4 in rat adipose tissues, indicating different modifications of oligosaccharide chains of the glucose transporter. However, different modifications of oligosaccharide chains in glucose transporters are sometimes observed in different cells and different tissues. For example, two species of GLUT1 with different electrophoretic mobilities were observed in rat brain, which contains various kinds of cells [2]. Such an example is also shown in this study; GLUT1 in 3T3-L1 cells exhibited lower electrophoretic mobility than GLUT1 in rat epididymal adipose tissues (Fig. 1*c*). Very similar to the modifications in GLUT1, GLUT4 in 3T3-L1 adipocytes was reported to migrate more slowly than that in rat adipocytes on SDS/PAGE [25]. This is exactly what was observed for expressed GLUT4 in 3T3-L1 cells and GLUT4 in rat adipocytes in the present study. Thus the different electrophoretic mobilities observed for GLUT4 are probably due to differences in the cell types in which the glucose transporter is expressed. On the other hand, exogenous expression of glucose transporters by using the expression vector does not necessarily induce a different carbohydrate modification from that of endogenous glucose transporter, since exogenous and endogenous GLUT1 in CHO cells exhibited the same mobility on SDS/PAGE, as previously observed [15,19]. Thus different carbohydrate modifications do not necessarily indicate incorrect folding of the expressed GLUT4, but are rather a result of the differences in the cell type in this study.

The reason why rat GLUT4 does not respond to insulin is not clear at present. However, it is not likely to be due to a small number of insulin receptors in CHO cells, since GLUT1 responds to insulin in the same cells. In addition, acquisition of a large insulin response precedes the increase in insulin receptor number during differentiation into 3T3-L1 adiocytes [24]. Thus there must be specific reasons for the unresponsiveness of expressed GLUT4 in relation to its intracellular localization and sorting processes. For a large insulin response. GLUT4 probably needs a proper cellular environment which fibroblasts lack but adipocytes have, such as GLUT4-specific intracellular vesicles [26]. Without this appropriate cellular environment, exogenous

GLUT4 might be stacked unphysiologically at some intracellular location and therefore unable to respond to insulin stimulation.

In this context, studies on the expression of GLUT4 in 3T3-L1 adipocytes and the effects of insulin on this expressed protein would be very interesting. When transfected 3T3-L1 fibroblasts were differentiated into adipocytes, the glucose transport activity increased by more than 5-fold (large response). However, we could not demonstrate any evidence that exogenous rat GLUT4 responds to insulin in 3T3-L1 adipocytes. This is partly due to the level of GLUT4 expression, because a large amount of endogenous mouse GLUT4 is also expressed after differentiation into adipocytes. The amount of endogenous mouse GLUT4 appears to be greater than that of the expressed exogenous rat GLUT4. This estimation is based on the assumption that the expression of mouse GLUT4 is not altered by co-expression of rat GLUT4, and that the expression of rat GLUT4 is not altered after differentiation into adipocytes. Since our three types of antibodies against rat GLUT4 could not distinguish mouse GLUT4 from rat GLUT4, we were not able to demonstrate whether or not the exogenous rat GLUT4 was translocated by insulin. In any case, it is apparent that some additional cellular factors are required for the acquisitions of a large insulin response in fibroblasts. One of these is probably the GLUT4-specific vesicle [26], and co-expression of these vesicle components would also be required. Elucidation of these cellular components is of great importance, not only in understanding the mechanisms of the large response of glucose transport activity to insulin, but also in understanding a crucial cellular function, i.e. protein sorting.

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