Development of the hormone-sensitive glucose transport activity in differentiating 3T3-L1 murine fibroblasts

Role of the two transporter species and their subcellular localization

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The development of a hormone-responsive glucose transport activity during differentiation of 3T3-L1 murine fibroblasts to an insulin-sensitive adipocyte-like phenotype was studied. Glucose transport activity was insensitive to insulin or insulin-like growth factor I (IGF-I) before differentiation, and was increased by 8–10-fold after differentiation by both insulin and IGF-I via their own respective receptors. In contrast, in undifferentiated cells insulin and IGF-I stimulated a large increase of [³H]thymidine incorporation into DNA via IGF-I receptors, indicating that undifferentiated 3T3-L1 cells are equipped with fully functioning hormone (IGF-I) receptors. Thus the previously described increase in expression of insulin receptors during differentiation cannot solely account for the development of hormone-sensitive glucose transport in the 3T3-L1 cell. The total glucose transport activity reconstituted from membrane fractions was increased by about 3-fold during differentiation. In differentiated cells, more than 80 % of the total reconstitutable glucose transport activity was detected in an intracellular compartment (200 000 g microsomes) as compared with about 20 % in undifferentiated cells. Immunoblots with specific antiserum confirmed previous reports indicating that the adipose tissue/muscle glucose transporter (GT3) was exclusively present in the differentiated cells, whereas the erythrocyte/brain glucose transporter (GT1) was detected in both differentiated and undifferentiated cells. Upon differentiation, GT1 was redistributed from plasma membranes to the intracellular compartment. In addition, the newly formed GT3 was predominantly found (> 80 % of total) in the microsomal fraction of differentiated cells. Both GT1 and GT3 appeared to be hormone-sensitive, since in differentiated cells insulin as well as IGF-I gave rise to their translocation from the intracellular compartment to the plasma membrane. These data suggest that, in addition to the specific expression of the GT3 transporter, the formation of a large pool of intracellular glucose transporters comprising both GT1 and GT3 contributes to the development of insulin sensitivity in the 3T3-L1 cell.

INTRODUCTION

The 3T3-L1 cell, a murine fibroblast cell line, differentiates to an adipocyte-like cell which synthesizes and stores triacylglycerols (Green & Kehinde, 1974). During differentiation of the cells, glucose transport becomes highly sensitive to insulin (Rosen *et al.*, 1978; Resh, 1982). Therefore the 3T3-L1 cell has been widely used as a model to study the molecular basis of insulin action on glucose transport.

Previous studies have suggested that the abundance of insulin receptors confers insulin sensitivity on the 3T3-L1 cell, since differentiation increased the number of insulin receptors per cell by about 20-fold (Rubin *et al.*, 1978). However, the time course of insulin receptor expression differed from that of the onset of the metabolic effects, in that the metabolic effects of insulin were detectable 1 day before the increase in receptor expression (Rosen *et al.*, 1978). In the present study, we used a different approach in order to elucidate whether expression of insulin receptors is sufficient for the development of insulin sensitivity in the 3T3-L1 cells. We compared the effects of insulin and insulin-like growth factor I (IGF-I) on glucose transport both before and after differentiation of the cells, for the following reason. The receptors for insulin and IGF-I have a high degree of sequence similarity (Ullrich *et al.*, 1985, 1986), and appear to possess the same potential to stimulate glucose transport (Lammers *et al.*, 1989). Unlike insulin receptors, IGF-I receptors are present in 3T3-L1 cells in approximately equal numbers

before and after differentiation (Smith *et al.*, 1988). Thus, if hormone receptor expression were the only factor conferring insulin sensitivity, and if IGF-I receptors were able to mediate a stimulation of glucose transport, one would anticipate that glucose transport in the undifferentiated 3T3-L1 cell can be stimulated by IGF-I. However, the present data failed to verify this assumption and indicate, therefore, that the expression of hormone receptors cannot be the only factor conferring insulin sensitivity in the 3T3-L1 cell.

In adipose and muscle tissue, glucose transporters are present in a large intracellular pool from which they are translocated to the plasma membrane in response to insulin (Cushman & Wardzala, 1980; Suzuki & Kono, 1980; Wardzala & Jeanrenaud, 1981). This effect can be demonstrated after homogenization of insulin-treated cells and separation into plasma membranes and a low-density microsomal fraction (Cushman & Wardzala, 1980; Suzuki & Kono, 1980). In order to assess the potential role of such an intracellular pool of glucose transporters in the development of insulin sensitivity in the 3T3-L1 cell, we studied the subcellular distribution of glucose transporters before and after differentiation by reconstitution of the transport activity into lecithin liposomes and by immunoblotting with specific antisera. The data show that, upon differentiation, a large pool of intracellular glucose transporters is formed.

It was demonstrated recently that 3T3-L1 cells contain two types of glucose transporters (James *et al.*, 1989; Kaestner *et al.*, 1989). The erythrocyte/brain-type glucose transporter (GT1) is

Abbreviations used: IGF-I, insulin-like growth factor I; GT1, erythrocyte/brain glucose transporter; GT3, adipose tissue/muscle glucose transporter.

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present in both differentiated and undifferentiated cells, whereas the muscle/adipose-type glucose transporter (GT3) is expressed only in differentiated cells. This finding has led to the hypothesis that expression of this tissue-specific transporter confers insulin sensitivity by a still unknown structural element in the sequence of the protein (James *et al.*, 1989; Kaestner *et al.*, 1989). Based on this hypothesis, one would expect that GT1, which is present in both differentiated and undifferentiated cells, is unresponsive to the hormone. In the present study, we tested this assumption by comparing the effects of insulin on the subcellular distributions of GT1 and GT3 in 3T3-L1 adipocytes. Since the results indicate that both transporters are hormone-sensitive, it is suggested that insulin sensitivity of the 3T3-L1 cell is not only conferred by the expression of the GT3 transporter, but also by some as yet unknown alteration in the machinery of the differentiated cell which leads to the formation of a large intracellular pool of glucose transporters.

MATERIALS AND METHODS

Cell culture

3T3-L1 fatty fibroblasts (Green & Kehinde, 1974) were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and were grown in Dulbecco's modified Eagle's medium (pH 7.4) containing 10% newborn calf serum, 20 mM-Hepes and 2 mM-glutamine. Undifferentiated cells were used 4 days after plating at a density of approx. 10 000 cells/cm². Cells were differentiated 2 days after confluence (7 days after plating) with a modification of the described protocol (Frost & Lane, 1985) by treatment with isobutylmethylxanthine (0.5 mM), dexamethasone (0.25 µM) and insulin (1 µg/ml) for 3 days, followed by treatment with insulin alone for 4 days. During differentiation, media were supplemented with fetal calf serum (10%) instead of newborn calf serum.

Assay of glucose transport activity

Glucose transport activity was determined with 2-deoxy[³H]glucose as described (Frost & Lane, 1985). Undifferentiated or differentiated cells were incubated for 2 h in serum-free culture medium supplemented with 1% bovine serum albumin. Thereafter the cells were washed three times with 1.5 ml of Krebs-Ringer/Hepes buffer (pH 7.4) containing 0.2% albumin, and were incubated for another 15 min in the same buffer supplemented with the indicated concentrations of insulin or IGF-I. The transport assay was started by addition of 2-deoxy[³H]glucose (1 µCi/sample) to a final concentration of 0.1 mM. Uptake was allowed to proceed for 10 min at 37 °C, and was terminated by cooling the cells on ice. The buffer was removed and cells were washed three times with ice-cold incubation buffer. The cells were lysed with 0.1% SDS and incorporated radioactivity was determined by scintillation counting in a water-compatible scintillation cocktail. The cell count was determined with parallel samples, and data were expressed per number of cells.

Assay of DNA synthesis

Undifferentiated or differentiated 3T3-L1 cells were washed once with Krebs-Ringer/Hepes buffer (0.2% albumin), incubated for 48 h in serum- and insulin-free medium supplemented with 1% albumin, and exposed to the indicated concentrations of insulin or IGF-I for an additional 17.5 h. Thereafter, cells were incubated with [³H]thymidine (1 µCi/well) for 2 h. The cells were washed three times with ice-cold buffer and were lysed in 0.1 M-NaOH. The lysates were precipitated with trichloroacetic acid (20%) and filtered with suction through glass fibre filters (Schleicher and Schuell, Dassel, Germany).

Filters were rinsed five times with ice-cold trichloroacetic acid (10%) and once with ethanol. After drying the filters, ³H incorporation into the precipitated material was assayed by liquid scintillation counting.

Homogenization and fractionation of 3T3-L1 cells

The cells were homogenized and fractionated according to a modification of a protocol previously developed for adipocytes (Joost *et al.*, 1988; Weber *et al.*, 1988). Differentiated 3T3-L1 adipocytes were washed twice with TES homogenization buffer (containing 20 mM-Tris, 1 mM-EDTA and 255 mM-sucrose) which had been equilibrated to 4 °C. The cells were scraped off the culture dishes with a rubber policeman and were homogenized in a 50 ml Potter-Elvehjem homogenizer (A. H. Thomas Scientific; cat. no. 3431-E25; specific clearance 0.15 mm) at room temperature with 15 handstrokes. The homogenate was centrifuged at 12 000 *g* and 4 °C (rotor JA20, 10 000 rev./min) for 15 min. The resulting pellet was layered on a 38% sucrose cushion and centrifuged for 60 min at 100 000 *g* (rotor SW27; 23 000 rev./min). Plasma membranes were collected from the surface of the sucrose cushion. The supernatant of the first centrifugation was spun at 200 000 *g* (rotor TI60, 49 000 rev./min) for 75 min and yielded a pellet consisting of intracellular membranes (low-density microsomes). In some experiments, a pellet of total membranes was prepared by a slightly modified procedure. Differentiated or undifferentiated 3T3-L1 cells were washed twice with Tris buffer (20 mM) containing sucrose (255 mM) and EDTA (1 mM) at 18 °C. The cells were scraped off the plates with a rubber policeman and were homogenized with 10 strokes of the Potter-Elvehjem grinder driven by a motor (1100 rev./min). Total membranes were prepared by centrifugation of the homogenate for 75 min at 200 000 *g*.

Reconstitution of glucose transport activity from membrane fractions

Glucose transport activity reconstituted into lecithin liposomes was assayed essentially as described by Robinson *et al.* (1982) with modifications as described previously (Schürmann *et al.*, 1989). Briefly, membrane fractions were solubilized with 20 mM-sodium cholate in Tris buffer (20 mM, pH 7.4). Excess detergent was removed by gel filtration, and the transporter solution was combined with lecithin liposomes which had been prepared by two 3 min sonications of a 16% solution of egg lecithin (Sigma, Deisenhofen, Germany). The samples were sonicated for 5 s, frozen at -80 °C, thawed, and sonicated again for 12 s. Transport was assayed at 37 °C with D-[U-¹⁴C]glucose, and rates were corrected for non-carrier-mediated uptake with L-[1-³H]glucose. The assay was stopped after 10 s, and samples were filtered on membrane filters ME 24 (Schleicher and Schuell; cat. no. 401780). The dried filters were immersed in a water-compatible scintillation cocktail and shaken for 2 h before liquid scintillation counting.

Antisera

Antiserum α-GT1 (Wheeler *et al.*, 1982) against the human erythrocyte glucose transporter GT1 was raised with transport protein purified essentially as described (Baldwin *et al.*, 1982), except that octyl thioglucoside was used for solubilization instead of octyl glucoside. Antiserum α-CT1 against the C-terminus of GT1 [peptide in one-letter code: (C)EELFHPLGADSQV (Haspel *et al.*, 1988)] was a gift from Dr. S. Cushman, NIH, Bethesda, MD, U.S.A. Antiserum α-CT3 against the muscle/adipose tissue glucose transporter GT3 was raised with a peptide corresponding to the C-terminus of this transporter (sequence in one-letter code: STELEYLGPDEND) coupled to keyhole-limpet haemocyanin. Cross-reactivity of the sera was

assessed by immunoprecipitation of glucose transporters and subsequent immunoblotting with a second serum. In these experiments, α -GT1 and α -CT1 exhibited full cross-reactivity, whereas no cross-reactivity between α -CT1 and α -CT3 was detected.

Immunoblotting of glucose transporters

Samples of the membrane fractions (40 μ g of protein) were separated by SDS/PAGE and transferred on to nitrocellulose sheets (Schleicher & Schuell) with a semi-dry blotting apparatus (Sartoblot II; Sartorius, Göttingen, Germany). Sheets were blocked overnight by incubation with buffer containing 0.05% (w/v) Tween 20 and 150 mM-NaCl, and were incubated with the antisera at a dilution of 1:200 for 2 h at room temperature. After three washes with buffer containing albumin (1%), sheets were incubated with 125 I-labelled Protein A (0.1 μ Ci/ml) for 2 h, washed thoroughly and autoradiographed for 1–4 days.

RESULTS AND DISCUSSION

Development of insulin sensitivity of glucose transport in 3T3-L1 cells upon differentiation

Previous studies have shown that glucose transport in 3T3-L1 fibroblasts is unresponsive to insulin and is stimulated by 6–10-fold after differentiation of cells to the adipocyte-like phenotype (Rosen *et al.*, 1978; Resh, 1982). As is illustrated in Fig. 1, this development of the hormonal response reflected a decrease in the basal transport rate as well as an increase in the maximal transport rate. Further, the Figure illustrates that 3T3-L1 fibroblasts were not absolutely unresponsive to insulin after confluence; insulin stimulated the deoxyglucose uptake by approx. 120% (basal, 0.14 ± 0.04 ; insulin, 0.29 ± 0.04 nmol/min per 10^6 cells; means $\pm$ S.E.M.; $n = 3$). In pre-confluent 3T3-L1 fibroblasts, in contrast, glucose transport was virtually unresponsive to insulin (basal, 0.41 ± 0.05 ; insulin, 0.53 ± 0.01 nmol/min per 10^6 cells; transport rates after differentiation of

Fig. 1. Development of insulin sensitivity in 3T3-L1 cells upon differentiation induced by insulin, dexamethasone and isobutylmethylxanthine

Murine 3T3-L1 fibroblasts were grown to confluence (1.3×10^4 cells/cm 2) and differentiation was induced 2 days later by addition of insulin, dexamethasone and isobutylmethylxanthine as described in the Materials and methods section. At the indicated day after confluence, the cells were incubated in the absence (○) or presence (●) of 100 nM-insulin for 15 min, and 2-deoxy[3 H]glucose uptake was assayed as described. The data represent means of duplicate samples.

corresponding cell samples: basal, 0.14 ± 0.02 ; insulin, 1.06 ± 0.11 nmol/min per 10^6 cells; $n = 4$). Consequently, in all experiments designed to look at the fibroblast state, cells were used before confluence when a density of approx. 10000 cells/cm 2 was reached.

Comparison of the effects of insulin and IGF-I on DNA synthesis and glucose transport in undifferentiated and differentiated 3T3-L1 cells

In contrast to glucose transport, thymidine incorporation into DNA was markedly stimulated by insulin in the undifferentiated 3T3-L1 fibroblasts (Fig. 2a). IGF-I produced a comparable

Fig. 2. Effects of insulin and IGF-I on DNA synthesis in undifferentiated and differentiated 3T3-L1 cells

(a) DNA synthesis in undifferentiated cells. Culture dishes of pre-confluent 3T3-L1 fibroblasts were incubated with the indicated concentrations of insulin (○) or IGF-I (●) for 17.5 h. [3 H]Thymidine was added, and 3 H incorporation into DNA was assayed after 2 h as described in the Materials and methods section. (b) DNA synthesis in differentiated cells. After confluence, the 3T3-L1 fibroblasts were subjected to the differentiation protocol as described in the Materials and methods section. The differentiated cells were incubated with the indicated concentrations of insulin (○) or IGF-I (●) for 17.5 h, and [3 H]thymidine incorporation was assayed as described. Data represent means of duplicate samples from a representative experiment repeated three times.

stimulation of DNA synthesis (Fig. 2a). Half-maximally effective concentrations of insulin were about two orders of magnitude higher than those of IGF-I, and were considerably higher than those required for insulin receptor occupation (Rubin *et al.*, 1978). Thus the effect of insulin on thymidine incorporation was probably mediated by binding of insulin to IGF-I receptors and not to insulin receptors. Nevertheless, the data demonstrate the presence of functioning IGF-I receptors, presumably capable of mediating insulin effects, in the undifferentiated 3T3-L1 fibroblasts. Fig. 2(b) illustrates the effects of insulin and IGF-I on thymidine incorporation into DNA in differentiated 3T3-L1 adipocytes. A large stimulation of DNA synthesis was observed in response to both hormones. As in the fibroblasts, in the

Fig. 3. Effects of insulin and IGF-I on glucose transport activity in undifferentiated and differentiated 3T3-L1 cells

(a) Glucose transport in undifferentiated cells. Pre-confluent 3T3-L1 fibroblasts were incubated with the indicated concentrations of insulin (○) or IGF-I (●) for 15 min, and uptake of deoxyglucose was assayed as described in the Materials and methods section. Data were corrected for non-carrier-mediated uptake and represent the cytochalasin B-inhibitable transport activity. (b) Glucose transport activity in differentiated 3T3-L1 cells. 3T3-L1 adipocytes were incubated in the presence of the indicated concentrations of insulin (○) or IGF-I (●) for 15 min, and uptake of deoxyglucose was assayed. Data represent means of duplicate samples from a representative experiment repeated three times.

differentiated cells about 100-fold higher concentrations of insulin than of IGF-I were required to stimulate the DNA synthesis (Fig. 2b), indicating that the effect of insulin was probably mediated by the IGF-I receptors.

Fig. 3 illustrates the concentration-dependent effects of IGF-I on glucose transport activity in 3T3-L1 fibroblasts and adipocytes as compared with those of insulin. In spite of the presence of functioning IGF-I receptors in the undifferentiated fibroblasts, glucose transport was virtually unresponsive to both IGF-I and insulin. In contrast, in differentiated 3T3-L1 adipocytes both insulin and IGF-I exerted a large stimulation of 2-deoxyglucose uptake (Fig. 3b). Surprisingly, the curves produced by IGF-I and insulin were virtually superimposable. Thus IGF-I appears to stimulate glucose transport via its own receptor rather than via insulin receptors, since IGF-I binds to insulin receptors with 70-fold lower affinity than does insulin itself (Smith *et al.*, 1988).

Taken together, the data presented in Figs. 2 and 3 indicate that expression of hormone receptors does not sufficiently explain the development of hormone sensitivity of glucose transport in 3T3-L1 cells upon differentiation. In undifferentiated cells, IGF-I receptors mediated large effects of IGF-I and of insulin on DNA synthesis. However, IGF-I failed to significantly stimulate glucose transport in these cells. Since the receptors for IGF-I were capable of mediating the full stimulation of glucose transport, as was clearly shown in the differentiated cells, the absence of insulin receptors cannot be the only reason for the insulin insensitivity of the undifferentiated 3T3-L1 cell.

Subcellular distribution of reconstitutable glucose transporters in undifferentiated and differentiated 3T3-L1 cells

According to the results presented in Fig. 1, the hormonal response in differentiated cells was in large part due to a decrease in basal glucose transport rates. It was conceivable, therefore, that differentiation had caused a re-distribution of transporters from the plasma membranes to a mobilizable intracellular pool typical for adipocytes (Cushman & Wardzala, 1980; Suzuki & Kono, 1980). Thus we studied the subcellular distribution of glucose transporters by reconstituting the transport activity into lecithin liposomes (Fig. 4) and by immunoblotting (see Fig. 6). Homogenates of cells were separated by differential centrifugation to yield plasma membranes and a microsomal fraction by modification of a protocol previously developed for adipocytes (Joost *et al.*, 1988; Weber *et al.*, 1988). As is illustrated in Fig. 4, in differentiated cells more than 80% of the total reconstituted glucose transport activity was found in the intracellular microsomes (low-density microsomes) as compared with about 20% in undifferentiated cells. Furthermore, the transport activity reconstituted from basal plasma membranes was lower in differentiated than in undifferentiated cells. These results are compatible with the conclusion that differentiation of the 3T3-L1 cell had induced the formation of a large intracellular pool of glucose transporters. Since the total reconstituted transport activity (sum of activity in plasma membranes and microsomes) was about 3-fold higher in the differentiated cells, a subcellular redistribution of transporters alone cannot account for the formation of the large intracellular transporter pool and additional synthesis *de novo* must be considered.

Expression and subcellular distribution of two glucose transporter species in undifferentiated and differentiated 3T3-L1 cells

Recently, 3T3-L1 cells have been shown to express at least two types of glucose transporters, the erythrocyte/HepG2 cell glucose transporter (GT1) and the muscle/adipose glucose transporter (GT3), which is exclusively found in insulin-sensitive tissues

Fig. 4. Subcellular distribution of the reconstitutable glucose transport activity in 3T3-L1 cells before and after differentiation

Undifferentiated 3T3-L1 fibroblasts (a) or differentiated 3T3-L1 adipocytes (b) were incubated for 15 min without hormone (Bs), in the presence of 100 nM insulin (Ins) or in the presence of 100 nM-IGF-I (IGF). Homogenates of the cells were separated by differential centrifugation to yield plasma membranes (PM) and a low-density microsomal fraction (LD). Glucose transport activity was reconstituted into lecithin liposomes as described in the Materials and methods section. The data represent means $\pm$ S.E.M. of quadruplicate samples from a representative experiment repeated two (fibroblasts) or three (adipocytes) times.

(James *et al.*, 1989; Birnbaum, 1989; Kaestner *et al.*, 1989; Charron *et al.*, 1989; Fukumoto *et al.*, 1989). In 3T3-L1 cells, GT3 is detected only after differentiation (James *et al.*, 1989; Kaestner *et al.*, 1989). In order to compare glucose transporters in 3T3-L1 fibroblasts and adipocytes, we used specific antisera against the two different glucose transporter isoforms. GT1 was detected with serum raised against the human erythrocyte glucose transporter (α -GT1) (Wheeler *et al.*, 1982) and against a peptide corresponding to the C-terminus of the erythrocyte/HepG2 cell glucose transporter [α -CT1 (Haspel *et al.*, 1988)]. GT3 was detected with serum against a peptide corresponding to the C-terminus of the muscle/adipose tissue glucose transporter (James *et al.*, 1989). As is illustrated in Fig. 5, the serum against GT1 (Figs. 5a and 5b) reacted with a broad immunoreactive band at about 50 kDa in Western blots of microsomes from the 3T3-L1 fibroblasts. In addition, the peptide-derived antiserum α -CT1 reacted with a band of higher molecular mass (90 kDa). We assume that this immunoreactivity is non-specific, since it was not detected with the α -GT1 antiserum. After differentiation of the cells, the mean apparent molecular mass of the glucose transporter GT1 as detected with both sera was slightly reduced to 48 kDa. This molecular mass shift was reproducible in five different membrane preparations. Therefore the glucose transporter protein GT1 appears to undergo a structural change during the differentiation of 3T3-L1 cells. At present, we can only speculate on the molecular basis of this alteration, which

Fig. 5. Immunochemical detection of the glucose transporters GT1 and GT3 in 3T3-L1 cells before and after differentiation

Proteins of the 200,000 g pellet from undifferentiated 3T3-L1 fibroblasts (F) or differentiated 3T3-L1 adipocytes (A) were separated by SDS/PAGE and electrophoretically transferred to nitrocellulose filters. As indicated, Western blots were prepared with antiserum raised against the erythrocyte/HepG2 cell glucose transporter (α -GT1, a and α -CT1, b) or against the muscle/adipose tissue glucose transporter (α -CT3, c) as described in the Materials and methods section.

Fig. 6. Immunochemical detection of the glucose transporters GT1 and GT3 in membrane fractions from undifferentiated and differentiated 3T3-L1 cells

Undifferentiated (a) or differentiated (b, c) 3T3-L1 cells were incubated in the absence or presence of 100 nM-insulin for 15 min, and were thereafter homogenized and fractionated as described. Abbreviations: BP, plasma membranes from basal cells; IP, plasma membranes from insulin-treated cells; BL, low-density microsomes from basal cells; IL, low-density microsomes from insulin-treated cells. Membrane fractions were separated by electrophoresis, transferred to nitrocellulose filters, and immunoassayed for GT1 (a and b, with serum α -CT1) or GT3 (c, with serum α -CT3).

might reflect a change in the primary sequence induced by alternative splicing or, more likely, a post-translational modification of the transporter.

Confirming previous results, Fig. 5(c) shows that the muscle/adipose tissue glucose transporter GT3 was newly formed during differentiation (James *et al.*, 1989; Kaestner *et al.*, 1989). The protein was not detectable in undifferentiated 3T3-L1 fibroblasts, but produced a large signal in the differentiated cells, comparable with that of GT1 (Figs. 5a and 5b). In contrast, the immunoreactivity of GT1 was slightly decreased during differentiation.

In order to assess the subcellular distribution of the two transporter isoforms before and after differentiation, and the effect of insulin thereon, Western blots of membrane fractions were prepared with the specific antisera α -CT1 and α -CT3. Fig. 6

Table 1. Subcellular distribution of immunoreactivity of glucose transporters GT1 and GT3 in undifferentiated and differentiated 3T3-L1 cells

Undifferentiated (fibroblasts) or differentiated (adipocytes) 3T3-L1 cells were incubated in the absence (Bs) or presence of 100 nM-insulin (Ins) or 100 nM-IGF-I (IGF) for 20 min, and were thereafter homogenized and fractionated as described. Membrane fractions (PM, plasma membranes; LD, low-density microsomes) were separated by electrophoresis, transferred on to nitrocellulose filters and immunoassayed for GT1 (with serum α -CT1) or GT3 (with serum α -CT3). The radioactivity incorporated into the respective bands was determined by scintillation counting. Data were normalized per total immunoreactivity in a set of membranes (sum of PM and LD), and represent means $\pm$ S.E.M. of four (differentiated cells) or means $\pm$ range of two (undifferentiated cells) independent experiments. N.D., not determined.

Transporter	Immunoreactivity (% of total)					
	PM			LD		
	Bs	Ins	IGF-I	BS	Ins	IGF-I
GT1 in fibroblasts	65.9 $\pm$ 2.3	66.8 $\pm$ 3.0	N.D.	34.1 $\pm$ 2.3	33.2 $\pm$ 3.0	N.D.
GT1 in adipocytes	35.3 $\pm$ 3.6	55.1 $\pm$ 2.7	57.0 $\pm$ 1.4	64.8 $\pm$ 3.7	44.9 $\pm$ 2.7	43.0 $\pm$ 1.4
GT3 in adipocytes	13.4 $\pm$ 1.8	26.5 $\pm$ 2.0	32.8 $\pm$ 3.6	86.6 $\pm$ 1.8	73.5 $\pm$ 2.0	67.2 $\pm$ 3.6

shows a representative immunoblot of GT1 (Figs. 6a and 6b) and GT3 isoform (Fig. 6c) in plasma membranes and intracellular microsomes for basal and insulin-treated 3T3-L1 fibroblasts (Fig. 6a) and adipocytes (Figs. 6b and 6c). The quantification of several independent experiments, including the effects of IGF-I, is given in Table 1. Before differentiation, the GT1 isoform was predominantly located in the plasma membranes. Conversely, after differentiation the major portion of GT1 was detected in the microsomes. Furthermore, more than 80% of the newly synthesized GT3 was found in the microsomes of differentiated cells. This subcellular distribution of glucose transporters corresponds reasonably well with that of the reconstituted glucose transport activity (Fig. 4). Thus, upon differentiation of 3T3-L1 cells, a large intracellular pool of glucose transporters is formed by redistribution of GT1 and by insertion of the newly synthesized GT3 isoform into this pool.

Fig. 6 and Table 1 show that both glucose transporter isoforms undergo the characteristic translocation in response to insulin. In addition, IGF-I produced virtually the same response as insulin. Thus the results demonstrate that both transporters are hormone-sensitive in the differentiated 3T3-L1 cell.

The finding that a tissue-specific glucose transporter is expressed during differentiation of 3T3-L1 cells had led to the hypothesis that the presence of this transporter confers insulin sensitivity on a cell by a still unknown structural element in the sequence of the protein (James *et al.*, 1989; Kaestner *et al.*, 1989). Alternatively, it has been suggested that it is the cellular machinery rather than the particular type of transporter which confers insulin sensitivity (Joost & Weber, 1989; Pilch, 1990), since both GT1 and GT3 are translocated in response to insulin in rat adipocytes. The results presented have demonstrated that GT1 is present (Fig. 5) and apparently unresponsive to insulin (Figs. 4 and 6; Table 1) in the undifferentiated fibroblasts. In the differentiated cell, in contrast, GT1 has become hormone-sensitive, as judged from its translocation in response to insulin (Fig. 6 and Table 1). In addition, the subcellular distribution of GT1 was altered by differentiation of the cells, contributing to the formation of the large intracellular transporter pool. These findings suggest that insulin sensitivity may not only be conferred by the expression of a transporter species, GT3, which is unique to insulin-sensitive tissues, but also by the modification of a 'insulin-insensitive' transporter GT1, or of its subcellular localization. Thus it cannot be excluded that differentiation of the 3T3-L1 cell produces the machinery which causes translocation of glucose transporters in response to insulin, regardless of the structure of the transporter species present.

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