Increased intracellular sequestration of the insulin-regulated aminopeptidase upon differentiation of 3T3-L1 cells

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In fat and muscle cells, the glucose transporter GLUT4 is sequestered in an intracellular compartment under basal conditions and redistributes markedly to the plasma membrane in response to insulin. Recently, we characterized a membrane aminopeptidase, designated IRAP (insulin-regulated aminopeptidase), that colocalizes with intracellular GLUT4 and similarly redistributes markedly to the plasma membrane in response to insulin in adipocytes. In contrast to GLUT4, IRAP is also expressed in 3T3-L1 fibroblasts, and this finding provided an opportunity to compare its subcellular distribution in fibroblasts and adipocytes. The relative amount of IRAP at the cell surface was measured by a cell surface biotinylation method. The portion of total IRAP at the cell surface in unstimulated adipocytes was 30 % of that in unstimulated fibroblasts. Upon insulin treatment the portion of IRAP at the cell surface was the same in fibroblasts and adipocytes, and was increased 1.8-fold in fibroblasts and 8-fold in adipocytes. A similar analysis of the distribution of the transferrin receptor (TfR), the paradigm for recycling plasma membrane receptors, revealed that the portions of the TfR at the cell surface in both the basal and insulin-treated states were almost unchanged upon differentiation, and that insulin caused an increase of about 1.6-fold in the amount of TfR at the cell surface. These results show that enhanced intracellular sequestration of IRAP occurs during adipogenesis, and that this effect underlies the larger insulin-elicited fold increase of IRAP at the cell surface in adipocytes.

INTRODUCTION

Insulin causes a rapid and marked increase in the amount of the glucose transporter GLUT4 at the cell surface in fat and muscle cells [1]. This effect is largely due to an increase in the rate of translocation of GLUT4 to the cell surface, from the intracellular sites where it is concentrated in the basal state [2]. Present evidence indicates that intracellular GLUT4 is located in both specialized vesicles and endosomes [3–5]. The increased rate of translocation is probably due to enhanced exocytosis of the specialized vesicles. However, it is also possible that the increased rate is, in part, due to more of the endosomal GLUT4 being incorporated into the vesicles that constitutively bud from the endosomes and traffic to the plasma membrane [3–5]. We and others have recently characterized a membrane aminopeptidase whose trafficking is very similar to that of GLUT4 (referred to here as IRAP, for insulin-regulated aminopeptidase; elsewhere designated vp165 and gp160) [6,7]. In unstimulated rat and 3T3-L1 adipocytes IRAP is largely, if not entirely, colocalized with GLUT4 in intracellular tubules and vesicles, as shown by subcellular fractionation [8–10], isolation of GLUT4 and IRAP-containing vesicles [8–10], immunofluorescence [10,11], and immunoelectron microscopy [12]. Like GLUT4, IRAP rapidly and markedly translocates to the cell surface in response to insulin treatment of adipocytes [8–11].

GLUT4 is primarily expressed in fat and muscle cells [1]. By contrast, IRAP has a much wider tissue distribution [6,10]. For example, whereas GLUT4 is normally expressed in 3T3-L1 cells only upon their differentiation into adipocytes, IRAP is also expressed in both the progenitor 3T3-L1 fibroblasts and the adipocytes [10]. In a number of studies GLUT4 has been expressed in 3T3-L1 and other fibroblasts by transfection, and its subcellular distribution and degree of translocation to the plasma membrane in response to insulin examined. Three of these studies concluded that GLUT4 is primarily intracellular in unstimulated fibroblasts, and that no translocation occurs in response to insulin [13–15]. A fourth study reported modest translocation in fibroblasts [a 3-fold increase in the plasma membrane], but did not determine the subcellular distribution of GLUT4 [16,17]. The first three studies thus indicate that fibroblasts and adipocytes both sequester GLUT4 intracellularly, but that only adipocytes exhibit significant insulin-stimulated translocation. A straightforward interpretation of these conclusions, which was proposed in [13], is that fibroblasts and adipocytes possess a similar mechanism for intracellular sequestration of GLUT4, but only adipocytes possess the mechanism for insulin-stimulated translocation.

The expression of IRAP in 3T3-L1 fibroblasts as well as 3T3-L1 adipocytes has allowed us to compare its subcellular distribution and insulin responsiveness in the two cell types without the complication of ectopic expression in the fibroblasts. In contrast to the findings for GLUT4, our results show that IRAP is more highly sequestered in adipocytes and that both cell types exhibit insulin-stimulated translocation of IRAP. Moreover, since our results show that fibroblasts and adipocytes have the same proportion of IRAP at the cell surface in the insulin-treated state, we conclude that the greater sequestration of IRAP in adipocytes underlies its larger degree of translocation in adipocytes.

EXPERIMENTAL

Antibodies

The antibodies against IRAP and GLUT4 used throughout were affinity-purified rabbit antibodies against the entire intracellular domain of IRAP and the carboxyl terminal 19 amino acids of

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; GLUT1 and GLUT4, glucose transporter isotypes; IRAP, insulin-regulated aminopeptidase; TfR, transferrin receptor.

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GLUT4. These have been characterized previously [6,18]. The antibody for the TfR was the murine monoclonal H68.4 against the cytoplasmic domain described in [19]; it was a generous gift from Dr. Ian Trowbridge of the Salk Institute.

Cell culture

3T3-L1 fibroblasts were cultured and differentiated into adipocytes as described previously [10]. 3T3-L1 fibroblasts were used at 2 days post confluence, while 3T3-L1 adipocytes were used at 8–12 days after the initiation of differentiation. The 3T3-L1 fibroblast cell line that expresses GLUT4 by transfection (IRGT-25) and the corresponding control cell line with vector alone (Neo-1) were cultured with 100 μg/ml (active concentration) of geneticin (Gibco-BRL). To induce maximal expression of GLUT4, these cells were treated for 48 h prior to use by the addition of 7 mM sodium butyrate and 100 μM $ZnCl_2$ to the culture medium as described previously [13]. Throughout, before use cells were placed in serum-free DMEM for 2 h.

Biotinylation of IRAP and TfR at the cell surface

Plates of cells in serum-free DMEM were treated with 1 μM insulin for 15 min or left in the basal state. In a study on the rate at which insulin causes IRAP to increase at the cell surface, which will be reported elsewhere, we have determined that the full increase occurs within 5 min in both fibroblasts and adipocytes and that there is no further change over the next 15 min. After the 15 min exposure to insulin, cell surface biotinylation, preparation of a lysate with nonionic detergent, and the isolation of IRAP by immunoadsorption, which was at least 90% complete, were carried out exactly as described in detail in [10]. This method involves rapid cooling of the cells to 4 °C in order to stop membrane trafficking, followed by reaction of the cells for 30 min with sulpho-NHS-LC-biotin (Pierce), a membrane-impermeant biotinylating reagent (5 ml of 1 mM reagent on a 10 cm plate). Under these conditions, with either fibroblasts or adipocytes, the concentration of the reagent decreased by only 5% over the 30 min period, as determined by spectrophotometric measurement at 270 nm of the concentration of the product *N*-hydroxysuccinimide in the medium. For isolation of the TfR, cell lysates derived from about 10^6 fibroblasts or adipocytes, prepared as described in [10], were incubated at 4 °C with 2 μg monoclonal antibody against TfR for 2 h, and then the immune complexes were collected by mixing with 10 μl protein G-Sepharose for 1.5 h. The TfR was released from the protein G-Sepharose by mixing at room temperature with 20 μl of SDS sample buffer containing 20 mM dithiothreitol and 8 M urea. Just before loading on the gel, the TfR was fully reduced by holding the SDS sample at 100 °C for 5 min. Comparison of the TfR content of the lysate with that of the lysate after immunoadsorption by immunoblotting showed that at least 90% of the TfR was immunoadsorbed under the conditions used.

Gel electrophoresis and blotting

The procedures for SDS gel electrophoresis and blotting for biotin with streptavidin-horseradish peroxidase conjugate and for IRAP and GLUT4 were as described in detail in [10]. In most of the experiments, the Immobilin P membrane was first blotted for biotin, and then it was stripped by treatment for 30 min at 50 °C with 2% SDS, 0.1% mercaptoethanol, 70 mM Tris/Cl, pH 6.8, followed by washing with 0.1% Tween 20, 150 mM NaCl, 20 mM Tris/Cl, pH 7.4 at room temperature, and re-probed with antibodies against IRAP or TfR. The TfR was blotted with the monoclonal antibody H68.4 at 3 μg/ml, followed by goat antibodies against mouse immunoglobulin conjugated to horseradish peroxidase (BioRad). Detection for all blots was by the enhanced chemiluminescence method (reagents from Pierce). In the case of both IRAP and TfR, the major biotinylated protein on the blot coincided in its mobility with the IRAP or TfR detected upon reprobing. The relative intensities of bands on each blot were quantitated by computer-enhanced video densitometry using the program NIH Image.

The key experiments (see Figures 1 and 2) required measurement of the relative biotin content per amount of IRAP or TfR for four samples, those from basal and insulin-treated fibroblasts and from basal and insulin-treated adipocytes. In order to facilitate visual comparison of the biotin signals and accurate measurement, the loads of the samples were adjusted so that the corresponding lanes of each contained approximately the same amount of IRAP or TfR. Moreover, each blot contained three or more loads ($\times 1$, $\times 1/2$, $\times 1/4$, etc.) of each sample, and this allowed the construction of a plot of the biotin signal versus the IRAP or TfR signal for each sample. Using this data, the biotin signal for each sample was normalized to an identical amount of IRAP or TfR; this normalization usually consisted of only small correction because the samples were usually well matched. The normalized biotin signals were then converted to relative biotin content per the same amount of IRAP or TfR by means of a plot of biotin signal versus biotin amount, which was generated from the several loads of the most heavily biotinylated samples. This step was included because the plot of biotin signal versus biotin amount often was somewhat non-linear with a downward curvature. The same method was used to measure the relative biotin content per amount of IRAP for the four samples in Figure 4 (basal and insulin-treated 3T3-L1 fibroblasts expressing GLUT4 or not).

RESULTS

Insulin-stimulated translocation of IRAP to the cell surface in 3T3-L1 fibroblasts and adipocytes

The relative amounts of IRAP at the cell surface of basal and insulin-treated 3T3-L1 fibroblasts and adipocytes were assessed by a biotinylation procedure. Proteins at the cell surface were biotinylated on exposed lysine residues with the membrane-impermeant reagent sulpho-NHS-LC-biotin. Subsequently, IRAP was isolated by immunoadsorption with antibodies against its cytoplasmic domain. Then, the relative biotin content of the IRAP was measured by quantitative blotting of the protein with streptavidin conjugated to horseradish peroxidase. In order to allow direct comparison of fibroblasts and adipocytes, which contain different amounts of IRAP (see legend of Figure 3), the loads on the blots were adjusted so that the amount of IRAP per lane was approximately the same.

Figure 1 presents the data from a representative experiment, which has been performed three times. Treatment of 3T3-L1 fibroblasts with insulin led to an increase of 1.8 ± 0.1 (S.E.M.) fold in the biotin content of the IRAP, whereas treatment of the 3T3-L1 adipocytes led to an increase of 8.0 ± 2.0-fold. Thus, the fold increase in cell surface IRAP in the adipocytes was considerably larger than in the fibroblasts.

This difference between fibroblasts and adipocytes could be due to either a smaller portion of the IRAP at the cell surface in the basal adipocytes or a larger portion of the IRAP at the cell surface in the insulin-treated adipocytes or a combination of both cases. The correct explanation was determined through comparison of the biotin contents of the IRAP in basal and insulin-treated adipocytes with the corresponding values for

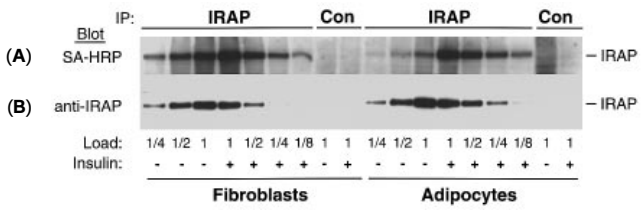

Figure 1 Biotinylation of cell surface IRAP in 3T3-L1 fibroblasts and adipocytes

Basal (−) and insulin-treated (+) 3T3-L1 fibroblasts and adipocytes were biotinylated at the cell surface, and samples of the cell lysates were immunoprecipitated with antibodies against IRAP or irrelevant antibodies (Con). Biotinylation was detected by blotting with streptavidin conjugated to horseradish peroxidase (SA-HRP (**A**)). The relative amounts of immunoprecipitated IRAP were determined by reprobing the blot with antibodies against IRAP (**B**). The loads of IRAP immunoadsorbate per lane were as shown, where load 1 was derived from 50% of a 10 cm plate of fibroblasts (approx. 2.5×10^6 cells) or 4% of a 10 cm plate of adipocytes (approx. 4×10^5 cells).

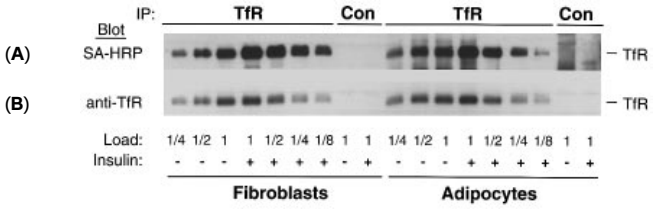

Figure 2 Biotinylation of cell surface TfR in 3T3-L1 fibroblasts and adipocytes

Basal (−) and insulin-treated (+) 3T3-L1 fibroblasts and adipocytes were biotinylated at the cell surface. Samples of the cell lysate were immunoprecipitated with antibodies against TfR or irrelevant antibodies (Con). Biotinylation was detected by blotting with streptavidin conjugated to horseradish peroxidase (SA-HRP (**A**)). The relative amounts of immunoprecipitated TfR were determined by reprobing the blot with antibodies against TfR (**B**). The loads of TfR immunoadsorbate per lane were as shown, where load 1 was derived from 10% of a 10 cm plate of fibroblasts (approx. 5×10^5 cells) or 5% of a 10 cm plate of adipocytes (approx. 5×10^5 cells).

fibroblasts. It is evident from the data in Figure 1 that the biotin content of IRAP in basal adipocytes was much less than that in basal fibroblasts, whereas the biotin contents of IRAP in insulin-treated adipocytes and fibroblasts were approximately the same. Thus, a smaller portion of the IRAP was located at the cell surface of adipocytes in the basal state, but upon insulin treatment the portion of IRAP at the cell surface was approximately the same for the two cell types. Consequently, upon differentiation of fibroblasts into adipocytes, a larger portion of the total IRAP was located intracellularly in the basal state. For the three experiments, the ratio of the portion of IRAP at the cell surface in adipocytes to that in fibroblasts averaged 0.30 ± 0.01 for cells in the basal state and 0.94 ± 0.17 for cells after insulin treatment.

Insulin-stimulated translocation of the TfR in 3T3-L1 fibroblasts and adipocytes

In order to compare the behaviour of IRAP with that of a protein known to cycle continuously from the plasma membrane through the endosomal system and back to the plasma membrane, the relative amounts of the TfR at the cell surface in basal and insulin-treated 3T3-L1 fibroblasts and adipocytes were determined by the same biotinylation method as used with IRAP. Figure 2 presents the data from a representative experiment, which has been carried out five times. Insulin treatment caused an increase of 1.8 ± 0.5-fold in the TfR at the cell surface in the fibroblasts and an increase of 1.4 ± 0.16-fold in the adipocytes. Moreover, the portions of the TfR at the cell surface in both the basal and insulin-treated adipocyte were approximately the same as those for the fibroblasts. The ratios of the portion of TfR at the cell surface in adipocytes to that in fibroblasts averaged 1.12 ± 0.15 and 0.98 ± 0.16 for the basal and insulin-treated states, respectively. Thus, unlike the case of IRAP, differentiation of the fibroblasts into adipocytes did not result in a larger portion of the TfR being intracellular in the basal state. This difference between the effect of differentiation on the portion of IRAP and of TfR at the cell surface shows that the enhanced intracellular sequestration of IRAP cannot be attributed to a general effect on recycling membrane proteins. The magnitude of the effect of insulin on TfR at the cell surface in 3T3-L1 adipocytes determined here by surface biotinylation agrees approximately with that from our earlier study, in which the TfR at the cell surface was measured by the binding of ^{125}I-labelled transferrin [20]. In the latter study insulin treatment caused a 1.7-fold increase in TfR at the cell surface.

Insulin-stimulated translocation of IRAP in 3T3-L1 fibroblasts expressing GLUT4

Since we previously found that IRAP and GLUT4 are colocalized in 3T3-L1 adipocytes [10], we expected that if GLUT4 were expressed in 3T3-L1 fibroblasts, it would be colocalized with IRAP in these cells as well. However, several studies have reported that upon expression in 3T3-L1 fibroblasts by transfection, GLUT4 is almost entirely intracellular, even after insulin treatment (see 'Introduction'). One potential explanation for this apparent difference between the subcellular distribution of IRAP and GLUT4 in fibroblasts is that the expression of GLUT4 somehow enhances the intracellular sequestration of IRAP. In order to examine this possibility, the effect of

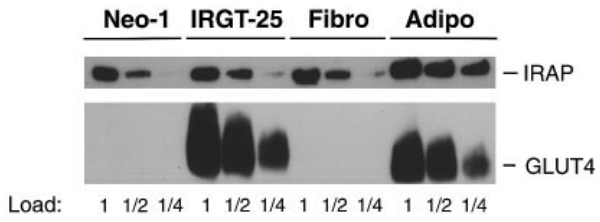

Figure 3 IRAP and GLUT4 contents of cell lines

SDS lysates were prepared from the fibroblasts cell lines containing control vector (Neo-1) or GLUT4 cDNA (IRGT-25) after induction with $ZnCl_2$ and butyrate, as well as from normal 3T3-L1 fibroblasts (Fibro) and adipocytes (Adipo). Samples were subjected to SDS gel electrophoresis and transferred onto Immobilon P. The upper and lower halves of the membrane were blotted for IRAP and GLUT4, respectively. The loads of lysate were as shown, where load 1 for the three types of fibroblast was 2% of a 10 cm plate [approx. 100000 cells] and load 1 for the adipocytes was 0.25% of a 10 cm plate (approx. 25000 cells). From the loads and the relative intensities of the signals, the GLUT4 content per cell of IRGT-25 was 45% of that of the 3T3-L1 adipocytes, and the IRAP content per cell of the three fibroblast lines was 10% of that of the 3T3-L1 adipocytes.

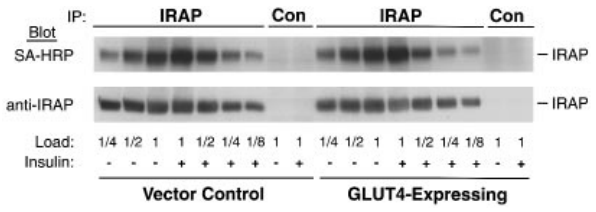

Figure 4 Biotinylation of cell surface IRAP in transfected 3T3-L1 fibroblasts expressing GLUT4

Stably transfected lines of 3T3-L1 fibroblasts expressing either GLUT4 or the vector control DNA (lines IRGT-25 and Neo-1, respectively) were induced with $ZnCl_2$ and butyrate, and then the basal (−) and insulin-treated (+) fibroblasts were biotinylated at the cell surface. Samples of the cell lysates were immunoprecipitated with antibodies against IRAP or irrelevant antibodies (Con). Biotinylation was detected by blotting with streptavidin conjugated to horseradish peroxidase (SA-HRP, (**A**)). The relative amounts of immunoprecipitated IRAP were determined by reprobing the blot with antibodies against IRAP (**B**). The loads of IRAP immunoadsorbate per lane were as shown, where load 1 was derived from 50% of a 10 cm plate of fibroblasts (approx. 2.5×10^6 cells).

GLUT4 expression in 3T3-L1 fibroblasts on the portion of IRAP at the cell surface was determined. The 3T3-L1 fibroblast cell line designated IRGT-25, which expresses GLUT4 under control of the metallothionein promoter, and the corresponding control line transfected with vector alone (designated Neo-1) were used. A previous study of IRGT-25 showed that its GLUT4 is predominantly intracellular in both the basal and insulin-treated state [13]. Upon induction with zinc ion and butyrate, IRGT-25 expressed approximately half the amount of GLUT4 per cell as did the 3T3-L1 adipocytes (Figure 3; see legend). The amount of IRAP in induced IRGT-25 was approximately the same as that in normal 3T3-L1 fibroblasts (Figure 3).

Figure 4 presents the analysis of the relative amounts of IRAP at the cell surface in basal and insulin-treated IRGT-25 and Neo-1, as determined by the cell surface biotinylation method. The biotin content of the IRAP from IRGT-25 in the basal states was the same as that of the IRAP from Neo-1. Thus, GLUT4 expression did not reduce the portion of IRAP at the cell surface. A repetition of this experiment showed the same result. In the experiment presented in Figure 4, insulin increased the amount of IRAP at the cell surface 1.7-fold for both IRGT-25 and Neo-1.

DISCUSSION

We have determined the fold increases of IRAP and TfR at the cell surface upon insulin treatment of 3T3-L1 fibroblasts and adipocytes, as well as the proportion of each protein present at the cell surface in adipocytes relative to that in fibroblasts. Although our method of measurement does not yield values for the actual percent of IRAP and TfR at the cell surface, these values can be estimated in the following ways. Yang et al. determined that 50% of the GLUT4 is at the cell surface in insulin-treated 3T3-L1 adipocytes, by a photoaffinity labelling method that compares the labelling of surface GLUT4 with that of total GLUT4 [21]. Previously, we compared the subcellular distribution of IRAP and GLUT4 in basal and insulin-treated 3T3-L1 adipocytes by simultaneously immunoblotting the plasma membrane and low density microsomal fractions from these cells for the two proteins [10]. This analysis showed that the distributions of IRAP and GLUT4 between the two fractions in the two states were similar. Thus, approx. 50% of the IRAP is also at the cell surface in insulin-treated 3T3-L1 adipocytes. Based on this value and our finding from biotinylation that IRAP increased 8-fold at the surface of adipocytes upon insulin treatment, we estimate that the percent of IRAP at the cell surface in basal 3T3-L1 adipocytes is 50%/8 or 6%. Moreover, since our results from biotinylation showed that the ratio of percent of IRAP at the cell surface in adipocytes to that in fibroblasts in the insulin-treated state was 0.97, we estimate that the percent of IRAP at the cell surface in insulin-treated fibroblasts is 50%/0.97 or 53%. Lastly, since the increase in IRAP at the cell surface of fibroblasts upon insulin treatment, as determined by biotinylation, was 1.8-fold, the estimated percentage of IRAP at the cell surface of basal fibroblasts is 53%/1.8 or 29%. It is worth noting that since an adipocyte contains 10 times as much IRAP as a fibroblast ([10] and Figure 3), the actual number of copies of IRAP at the cell surface of a basal adipocyte is larger than that of a basal fibroblast.

In the case of the TfR, we have previously determined by means of [^{125}I]transferrin binding to surface and total receptors that 30% of the TfR is at the cell surface in insulin-treated 3T3-L1 adipocytes [20]. From this value and the relative proportions of TfR at the cell surface determined in this study, in the same way as described above for IRAP, we estimate the percentages of TfR at the cell surface in basal 3T3-L1 adipocytes and basal and insulin-treated 3T3-L1 fibroblasts are 21, 17 and 30%, respectively.

Table 1 presents the values of the insulin-stimulated increase and the percentages at the cell surface for IRAP and TfR, together with corresponding values in the literature for GLUT1 and GLUT4. The values for GLUT1 in fibroblasts and adipocytes and for GLUT4 in adipocytes were measured by Yang et al. using the photoaffinity labelling method [21]. As noted earlier, 3T3-L1 fibroblasts do not normally express GLUT4 so that data for it must come from transfected fibroblast lines. There has been one measurement of the fold increase in GLUT4 in 3T3-L1 fibroblasts, which was made by measuring the binding of antibodies against a myc epitope to the surface of 3T3-L1 fibroblasts that express GLUT4 with the myc epitope in its extracellular domain [16]; this value is given in Table 1 (but see below). There have been no quantitative determinations of the percent of GLUT4 at the cell surface in 3T3-L1 fibroblasts (but see below).

In 3T3-L1 fibroblasts TfR, IRAP, GLUT1 and GLUT4 each increase approximately 2-fold at the cell surface in response to insulin. In the adipocytes the TfR increases 1.4–1.7-fold, but the other proteins exhibit larger increases, from 4.5-fold for GLUT1 to 11-fold for GLUT4 (Table 1). Previously, we showed that in adipocytes the 1.7-fold elevation in TfR on the cell surface is due to a 1.7-fold stimulation in the rate constant for recycling of the TfR from the endosomes to the plasma membrane [20]. A reasonable extrapolation from this previous finding is that this effect of insulin is on the rate constant for vesicular trafficking from the endosomes to the plasma membranes, and that therefore other proteins largely located in the endosomes will increase approximately 2-fold at the plasma membrane in response to insulin. Thus, the almost uniform 2-fold increase in the amounts of the four proteins at the surface of the 3T3-L1 fibroblasts can be explained by an insulin effect of this magnitude on the rate constant for recycling from the endosomes. This explanation assumes that the intracellular TfR, IRAP, GLUT1 and GLUT4 are primarily in endosomes in the fibroblasts.

The larger and varied fold elevations in the amounts of GLUT1, IRAP and GLUT4 at the cell surface of the adipocytes in response to insulin must have an alternate explanation. For both GLUT1 and IRAP, the basis for the larger effect in adipocytes versus fibroblasts lies entirely in greater intracellular

Table 1 Subcellular distribution of membrane proteins in 3T3-L1 fibroblasts and adipocytes

The fold increase at the cell surface in response to insulin (I/B), together with the percentages of the protein at the cell surface in the basal (B) and insulin (I) state are given.

	Fibroblast			Adipocyte		
Protein	I/B	B	I	I/B	B	I
TfR*	1.8	17	30	1.4	21	30
GLUT1†	2.3	25	57	4.5	10	45
IRAP*	1.8	29	53	8.0	6	50
GLUT4†	3.4‡	–	–	11	4.5	51

* I/B values are the ones measured in this study. Values of the percentages are estimated as described in the 'Discussion'.

† From Ref. [21]. The values for GLUT1 in fibroblasts are those for fibroblasts in the confluent state. In the sparse state 95% of the GLUT1 is at the cell surface in the basal state [21].

‡ This value from Ref. [16], for fibroblasts expressing myc-tagged GLUT4 through transfection.

sequestration of the protein in the basal state. Upon insulin treatment, the portion of both GLUT1 and IRAP at the cell surface is approximately the same in fibroblasts and adipocytes. The explanation for the enhanced intracellular sequestration in the adipocytes remains to be determined. One reasonable hypothesis is that the 3T3-L1 adipocytes possess specialized secretory vesicles in which substantial portions of the IRAP and GLUT4, as well as some of the GLUT1, are sequestered, and that 3T3-L1 fibroblasts lack these. Consistent with this proposal, recent studies characterizing the intracellular tubules and vesicles containing GLUT4 [4] and IRAP [12] in 3T3-L1 adipocytes have concluded that in the basal state approx. 60% of each is in specialized vesicles and 40% in recycling endosomes. Evidence of a completely different type, for a unique sequestration mechanism in adipocytes, comes from the analysis of the subcellular distribution of a GLUT4 mutant lacking its dileucine internalization motif [5]. In 3T3-L1 adipocytes, this mutant is predominantly located in intracellular vesicles, whereas in fibroblasts, a larger portion of it is at the cell surface.

If GLUT4 behaves the same way as GLUT1 and IRAP upon differentiation of 3T3-L1 cells, then the percentage of GLUT4 at the cell surface in insulin-treated 3T3-L1 fibroblasts should be approximately the same as that in adipocytes, which is 50%. In fact, results in the literature generally indicate that less than 50% of the GLUT4 expressed by transfection in 3T3-L1 fibroblasts is at the cell surface after insulin treatment; moreover, these studies disagree on whether insulin causes translocation of GLUT4 to the cell surface. Haney et al. examined the distribution of GLUT4 in 3T3-L1 fibroblasts by immunofluorescence, immunoelectron microscopy, subcellular fractionation and effect on glucose transport activity [13]. Although these methods of analysis were either semi-quantitative or indirect (effect on transport activity), the data indicate that GLUT4 is primarily intracellular in the basal state, and moreover, that there is no detectable translocation to the cell surface in response to insulin. GLUT4 has also been expressed by transfection in NIH 3T3 fibroblasts and Chinese hamster ovary (CHO) cells, cell types in which its behaviour is likely to be the same as in 3T3-L1 fibroblasts [14,15]. In these studies GLUT4 was also found to be primarily intracellular and not to translocate to the plasma membrane in response to insulin, even in cell lines overexpressing the insulin receptor, although again the methods for analysis of GLUT4 distribution were semi-quantitative or indirect. In contrast, as described above in part, Ishii et al. and Kanai et al. expressed GLUT4 tagged with the myc epitope in 3T3-L1 fibroblasts, NIH 3T3 cells and CHO cells, and found by a quantitative assay that insulin elicited an approximately 3-fold increase in the amount at the cell surface in each cell type [16,17]. This latter study, however, did not determine the overall distribution of GLUT4 in the basal state and after insulin treatment by a method such as photoaffinity labelling of intact and permeabilized cells [21]. One way to reconcile the conflicting conclusions about the insulin responsiveness of GLUT4 in fibroblasts from the various studies is to assume that only some small percentage of the GLUT4 is at the cell surface even after insulin treatment and that the semi-quantitative and indirect methods would have therefore missed its insulin-elicited increase. On the other hand, GLUT4 may well behave differently in the various transfected cell lines due to differences in cell types, transfection vectors, levels of GLUT4 expression, cell growth conditions, and extents of cell confluency. Definite resolution of these issues would require quantitative measurements of the percent of GLUT4 at the cell surface in the basal and insulin states for the transfected cell lines. However, the weight of evidence does suggest that GLUT4 ectopically expressed in fibroblasts is much more intracellular than IRAP, and this raises the possibility of a unique and unrepresentative subcellular distribution of GLUT4 in fibroblasts.

The considerations given above indicate the value of a quantitative analysis of the distribution of endogenous insulin-responsive membrane proteins in 3T3-L1 cells. Our comparison of the behaviour of IRAP in 3T3-L1 fibroblasts and adipocytes has shown for this protein that differentiation leads to enhanced intracellular sequestration, which in turn underlies the large fold increase at the cell surface in adipocytes in response to insulin. A challenge now is to delineate the mechanism of sequestration, including the identification of components thereof. It seems likely that one of these will be a protein interacting with the cytoplasmic domain of IRAP. The results of this study suggest that this protein is present in adipocytes, but not fibroblasts.

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