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Overexpression of the glucose transporter GLUT4 in adipose cells interferes with insulin-stimulated translocation

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Abstract In adipose cells, insulin induces the translocation of GLUT4 by stimulating their exocytosis from a basal intracellular compartment to the plasma membrane. Increasing overexpression of a hemagglutinin (HA) epitope-tagged GLUT4 in rat adipose cells results in a roughly proportional increase in cell surface HA-GLUT4 levels in the basal state, accompanied by a marked reduction of the fold HA-GLUT4 translocation in response to insulin. Using biochemical methods and cotransfection experiments with differently epitope-tagged GLUT4, we show that overexpression of GLUT4 does not affect the intracellular sequestration of GLUT4 in the absence of insulin, but rather reduces the relative insulin-stimulated GLUT4 translocation to the plasma membrane. In contrast, overexpression of GLUT1 does not interfere with the targeting of GLUT4 and vice versa. These results suggest that the mechanism involved in the intracellular sequestration of GLUT4 has a high capacity whereas the mechanism for GLUT4 translocation is readily saturated by overexpression of GLUT4, implicating an active translocation machinery in the exocytosis of GLUT4.

Key words: GLUT4; Insulin; Adipose cell; Overexpression; Translocation; Exocytosis

1. Introduction

GLUT4 glucose transporters are found in adipose and muscle cells, and are responsible for the glucose uptake regulated by insulin (reviewed in [1,2]). In adipose cells, GLUT4 are constantly recycling between intracellular compartments and the plasma membrane [3–6]. In the basal state, the majority of the GLUT4 is located in an intracellular tubulo-vesicular compartment [3,5,7]. Insulin causes an increase in the rate of exocytosis of GLUT4-containing vesicles, resulting in a rapid shift in the steady-state distribution of GLUT4 to the plasma membrane [3–5]. Rat adipose cells also express relatively low amounts of GLUT1 [8]. While GLUT4 are efficiently sequestered inside the cell in the absence of insulin, a greater proportion of GLUT1 are located in the plasma membrane [8]. However, despite their different steady-state distributions, both isoforms appear to cycle between intracellular compartments and the cell surface [6]. The extent to which the trafficking pathways of GLUT4 and GLUT1 overlap remains debated [9,10].

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Abbreviations: HA, hemagglutinin; PCR, polymerase chain reaction

Overexpression of GLUT4 in adipose [11] and muscle tissue [12–14] of transgenic mice, as well as in isolated rat adipose cells by transfection [15], has been reported to result in increased cell surface levels of GLUT4 in both the basal and insulin-stimulated states. In addition, in both adipose and muscle tissues, a drastic reduction in the fold GLUT4 translocation response to insulin was observed [11,14]. It was hypothesized that this effect was due to saturation of the sorting/retention mechanism responsible for the intracellular sequestration of GLUT4 in the basal state. However, after studying the protein composition of immunoadsorbed GLUT4-containing vesicles from adipose cells of these transgenic mice, it was concluded that the capacity of the vesicles appeared to be sufficient to incorporate the overexpressed GLUT4 [16]. Furthermore, according to subcellular fractionation studies of internal membranes, the sedimentation coefficients of GLUT4-containing vesicles from adipose cells of transgenic and non-transgenic mice were identical, suggesting that the specificity of the intracellular targeting of GLUT4 was not affected by the overexpression of the protein [16].

The aim of our study was to examine the effects of overexpression on the subcellular distribution of epitope-tagged GLUT4 in transfected rat adipose cells. Furthermore, we studied the interaction of GLUT1 and GLUT4 by coexpressing both glucose transporters in the same cells. Our results indicate that overexpression of GLUT4, but not GLUT1 interferes with the insulin-stimulated translocation of GLUT4 to the plasma membrane rather than the intracellular retention of GLUT4 in the basal state.

2. Materials and methods

2.1. Vectors, antibodies

The cDNAs of human GLUT1 and GLUT4 were gifts from Dr. G.I. Bell (University of Chicago). The expression vector pCIS2 was a gift from Dr. C. Gorman (Genentech). Anti-hemagglutinin (HA) antibodies (HA.11) were from Berkeley Antibody Co. Rabbit anti-GLUT4 antiserum (1154p) was kindly provided by Hoffmann-La Roche (Nutley, NJ).

2.2. Construction of HA-GLUT1

An *Eco*RV restriction site was introduced into the cDNA of GLUT1, thereby changing serine-55 in the first exofacial loop to aspartic acid. Site-directed mutagenesis was performed using the polymerase chain reaction (PCR)-based Quick-Change method (Stratagene). The primer (sense strand) was 5′-CACCGCTATGGGGAGGATATCCTGCCCACCACG-3′ (mismatches are underlined). The integrity of the mutated GLUT1 sequence was verified by dideoxynucleotide sequencing. An oligonucleotide (sense strand: 5′-TACCCATACGACGTGCCAGACTACGCC-3′) encoding the HA tag (YPYDVPDYA) was ligated into the *Eco*RV site and its orientation was verified by dideoxynucleotide sequencing. The *Sal*I/*Kpn*I fragments of the cDNAs for HA-GLUT1 and GLUT1 were then subcloned into the pCIS2 expression vector.

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2.3. Construction of HA-GLUT4 and FLAG-GLUT4

The construction of HA-GLUT4 has been described previously [17]. An oligonucleotide (sense strand: 5′-TGACTACAAAGACGATGACGATAAAGC-3′; antisense strand: 5′-TCAGCTTTATCGTCATCGTCTTTGTAGTCC-3′) encoding the FLAG tag (DYKDDDDK) was ligated into the unique *Bsu*36I site of GLUT4/pCIS2 and its orientation was verified by dideoxynucleotide sequencing. Both epitope tags, HA and FLAG, are located between Pro-66 and Asp-67 in the first exofacial loop of GLUT4.

2.4. Cell culture, transfection of rat adipose cells, and cell surface antibody binding assay

Preparation of isolated rat epididymal adipose cells from male rats (CD strain, Charles River Breeding Laboratories, Inc.) has been described previously [17]. The electroporation of rat adipose cells and the cell surface antibody binding assay were performed as described [15]. Briefly, isolated primary rat adipose cells ($\sim 10^6$ cells/sample) in Dulbecco's modified Eagle's medium were electroporated (3 square wave pulses of 12 ms, 500 V/cm) in the presence of 0.25 mg/ml carrier DNA (sheared herring sperm DNA, Boehringer Mannheim) and expression plasmid (1.25–12.5 μg/ml). After culturing in Dulbecco's modified Eagle's medium for 4–24 h at 37°C, 5% CO_2, the cells were stimulated with insulin (67 nM, 1×10^5 μU/ml) for 30 min at 37°C. Subcellular trafficking of GLUT4 was then stopped by addition of KCN to a final concentration of 2 mM and the cells were incubated for 1 h with a monoclonal anti-HA antibody (1:1000 dilution). After removal of excess antibody, 0.1 μCi of [^{125}I]sheep anti-mouse antibody (Dupont) was added and the cells were incubated for 1 h. Finally, the cells were spun through dinonylphthalate oil [18] to remove unbound labeled antibodies and the cell surface-associated radioactivity was counted in a γ-counter. The resulting counts were normalized to the lipid weight of the samples [18]. Antibody binding assays were performed in duplicate or quadruplicate. The values obtained for pCIS2-transfected cells were subtracted from all other values to correct for non-specific antibody binding.

2.5. Western blot analyses of HA-GLUT4

Western blot analyses of HA-GLUT4 were performed with a monoclonal anti-HA antibody (1:1000 dilution) and a polyclonal antiserum against the C-terminus of GLUT4 (1:4000 dilution). HA-GLUT4-transfected rat adipose cells were harvested after 24 h of protein expression and total cellular membranes were prepared as described previously [19]. The resulting membranes were separated by SDS-PAGE and transferred onto nitrocellulose filters, and the filters were then incubated with 0.1 μCi/ml [^{125}I]sheep anti-mouse antibodies, or 0.1 μCi/ml [^{125}I]protein A, respectively. Quantitation of the blots was performed using a PhosphorImager (Molecular Dynamics).

2.6. Determination of transfection efficiency

Rat adipose cells were transfected with various amounts of expression plasmid (0.5–5 μg DNA/cuvette) for HA-GLUT4 and cultured for 24 h at 37°C, 5% CO_2. After stimulation with insulin (67 nM, 30 min), the cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) in 0.15 M phosphate-buffered saline (PBS), pH 7.4, as described previously [7]. The fixed cells were then blocked for 45 min in PBS containing 1% bovine serum albumin (BSA) and stained using a monoclonal anti-HA second antibody (HA 1.1, 1:100 in PBS/1% BSA; 1 h) and a rhodamine-coupled anti-mouse antibody (1:100 in PBS/1% BSA; 1 h; Jackson ImmunoResearch). Finally, the cell surface-bound rhodamine conjugate was visualized by fluorescence microscopy. In each experiment, ~500 randomly selected cells were analyzed for HA-associated fluorescence. From three separate experiments the transfection efficiency was estimated to be ~10%.

3. Results

3.1. Overexpression of epitope-tagged GLUT4 and GLUT1 in isolated rat adipose cells

The primarily expressed GLUT isoform in adipose cells is GLUT4. However, these cells also contain GLUT1 which accounts for about 10% of the total glucose transporters [8]. To study the effects of overexpression of these glucose transporters in rat adipose cells on the cell surface levels of GLUT1 and GLUT4, we transfected these cells with HA epitope-tagged GLUT1 (HA-GLUT1) and GLUT4 (HA-GLUT4) [15]. The results of these experiments are illustrated in Fig. 1. As shown, increased amounts of expression plasmid and/or prolonged protein expression time resulted in an increase in cell surface HA-GLUTs in both the basal and insulin-stimulated states. However, whereas insulin stimulation of HA-GLUT4-transfected cells resulted in a marked increase in cell surface HA-GLUT4, a much smaller insulin effect was observed on GLUT1 in HA-GLUT1-transfected cells. Moreover, as described previously [15], the insulin response of HA-GLUT4 appeared to be dependent on the amount of HA-GLUT4 expressed. Whereas at low levels of protein expression insulin stimulation resulted in a ~10-fold increase in cell surface HA-GLUT4, the observed insulin response decreased to <2-fold at high expression levels. In contrast, the much smaller cell surface HA-GLUT1 response to insulin appeared to be unaffected by an increase in the protein expression levels. According to Western blot analyses using an anti-HA antibody, both proteins, HA-GLUT1 and HA-GLUT4, were expressed at similar levels (data not shown).

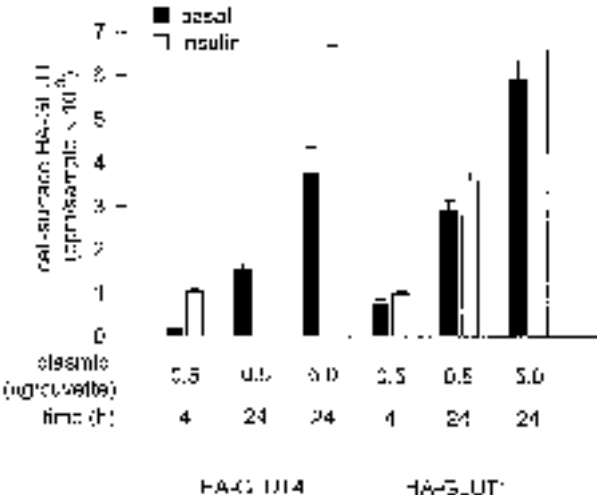

Fig. 1. Cell surface expression of HA-GLUT4 and HA-GLUT1 in rat adipose cells. Isolated cells were transfected with 0.5 μg or 5 μg plasmid/cuvette and cultured for 4 h or 24 h. After harvesting, the cells were incubated without (basal) or with 67 nM insulin for 30 min, and the cell surface levels of HA-epitope-tagged glucose transporters were determined using an antibody binding assay. Results are the means ± S.E.M. of the mean values obtained from duplicate to quadruplicate determinations in at least three independent experiments.

3.2. Quantitation of HA-GLUT4 overexpression in rat adipose cells

To determine the magnitude of overexpression of HA-GLUT4, rat adipose cells were transfected with various amounts of HA-GLUT4 expression plasmid and cultured for 24 h. HA-GLUT4 and endogenous GLUT4 were then quantitated by Western blots of total cellular membranes. The results are illustrated in Fig. 2. The epitope tagged HA-GLUT4 appeared as a double band where the upper band migrated with a slightly higher M_r than the endogenous GLUT4 in SDS-PAGE thus allowing the simultaneous detection of both recombinant and endogenous GLUT4 by using an antiserum directed against the carboxy-terminus of

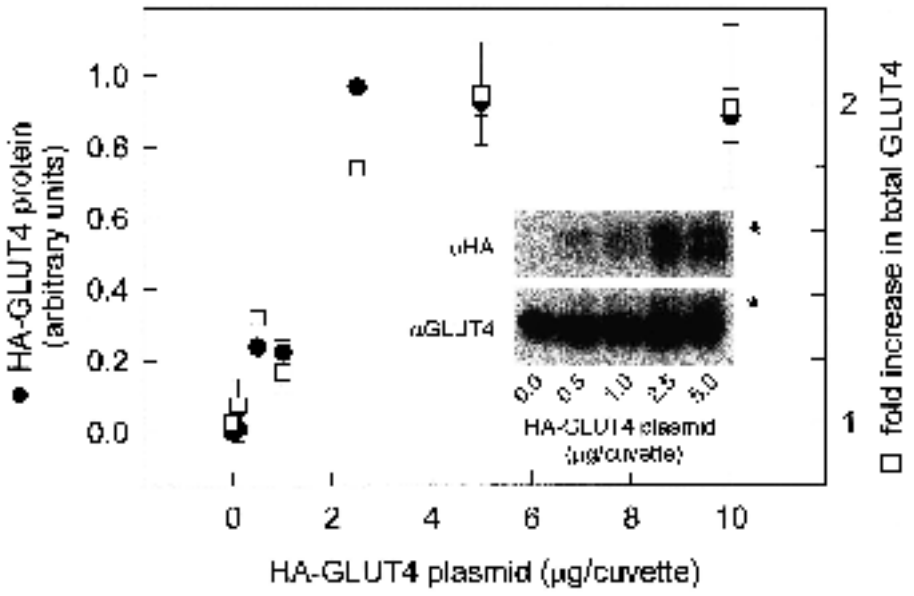

Fig. 2. Relationship between HA-GLUT4 expression plasmid concentration and expression levels. Isolated cells were transfected with increasing amounts of expression plasmid and cultured for 24 h. After harvesting, the amounts of HA-GLUT4 and total GLUT4 were determined by Western blot analyses of total cellular membranes (inset: an asterisk denotes the position of the upper HA-GLUT4 band). The obtained protein expression levels were normalized to the mean maximum value. Results are the means ± S.E.M. of the mean values obtained from duplicate to quadruplicate determinations in three independent experiments.

GLUT4. Expression of HA-GLUT4 appeared to be roughly linear for plasmid concentrations between 0.1 and 2.0 μg DNA/cuvette. However, higher concentrations of expression plasmid did not result in a further increase in the amount of HA-GLUT4 expressed. Under conditions of maximal HA-GLUT4 expression, the total amount of GLUT4 (i.e. endogenous and recombinant GLUT4) was increased 2-fold compared to cells transfected with the empty expression vector. Taking into account the transfection efficiency (see Section 2) of ~10% [15], maximal expression of HA-GLUT4 resulted in a 20-fold overexpression of GLUT4 per transfected cell.

3.3. Cell surface expression of HA-GLUT4 in transfected rat adipose cells

Fig. 3 illustrates the relationship between the amount of HA-GLUT4 expressed and the respective cell surface levels of HA-GLUT4 in transfected rat adipose cells. Increasing amounts of cellular HA-GLUT4 resulted in a concomitant increase in cell surface levels of HA-GLUT4 in both the basal and insulin-stimulated states (Fig. 3A). However, whereas the increase in basal cell surface HA-GLUT4 appeared to be almost linear with respect to the HA-GLUT4 protein levels, the increase in cell surface HA-GLUT4 in the insulin-stimulated state leveled off before half-maximal protein expression was reached. As a result, the observed insulin response decreased sharply from ~4-fold at low levels of HA-GLUT4 expression to ~1.5-fold at half-maximum protein expression (Fig. 3B). At the highest expression levels, the cell surface levels of HA-GLUT4 in the basal state were almost equal to the cell surface levels in the presence of insulin.

Fig. 3C shows the relationship between cell surface HA-GLUT4 and total expressed cellular HA-GLUT4 when the former is calculated as a relative proportion of the latter; the need for normalization of the data among experiments and between cell surface antibody binding and Western blot-

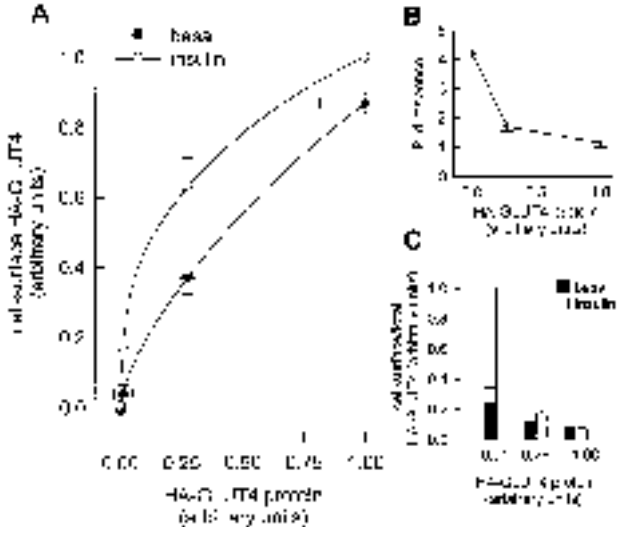

Fig. 3. Relationship between HA-GLUT4 expression level and cell surface distribution. Isolated cells were transfected with increasing amounts of expression plasmid (0.1–5 μg plasmid/cuvette) and cultured for 24 h. After harvesting, the cells were incubated without (basal) or with 67 nM insulin for 30 min, and the cell surface levels of HA-GLUT4 were determined using an antibody binding assay. In parallel, the respective expression levels of HA-GLUT4 were determined by Western blot analyses of total cellular membranes. A: Data are plotted as cell surface HA-GLUT4 against HA-GLUT4 expression level. B: Data are expressed as fold cell surface HA-GLUT4 insulin responses and plotted against HA-GLUT4 expression level. C: Data are expressed as relative proportions of HA-GLUT4 expression found at the cell surface and plotted against HA-GLUT4 expression level. Results are the means ± S.E.M. of the mean values obtained from duplicate to quadruplicate determinations in three independent experiments.

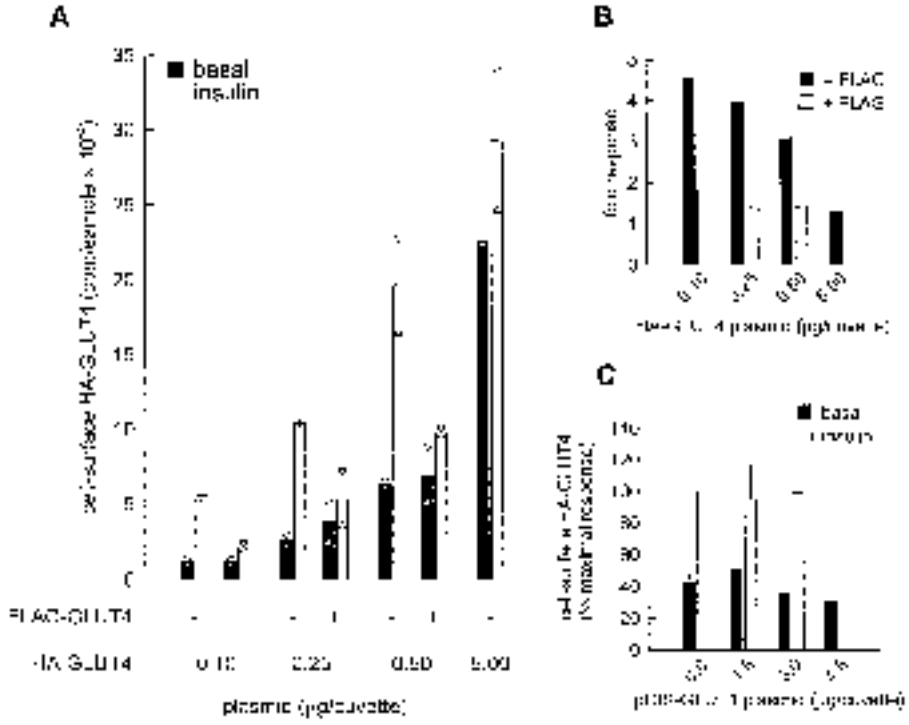

Fig. 4. Coexpression of HA-GLUT4 and FLAG-GLUT4 (A, B), and HA-GLUT4 and GLUT1 (C). A, B: Isolated cells were cotransfected with 0 (−) or 5 (+) μg FLAG-GLUT4 plasmid/cuvette and increasing amounts of expression plasmid for HA-GLUT4. After 24 h, the cells were incubated without (basal) or with 67 nM insulin for 30 min, and the cell surface levels of HA-GLUT4 were determined using an antibody binding assay. Data are plotted as cell surface HA-GLUT4 (A) and are also expressed as fold cell surface HA-GLUT4 insulin responses in the absence (−) and presence (+) of FLAG-GLUT4 (B). Results are the means of the mean values (△, ▽) obtained from at least duplicate determinations in two independent experiments. C: Isolated cells were cotransfected with 0.5 μg HA-GLUT4 plasmid/cuvette and increasing amounts of expression plasmid for GLUT1. After 24 h, the cells were incubated without (basal) or with 67 nM insulin for 30 min, and the cell surface levels of HA-GLUT4 were determined using an antibody binding assay. Results are the means ± S.E.M. of the mean values obtained from duplicate to quadruplicate determinations in three independent experiments.

ting does not allow determination of absolute proportions. When the total cellular expression level of HA-GLUT4 was increased by two orders of magnitude, the relative proportion of cell surface HA-GLUT4:total HA-GLUT4 was decreased ∼4-fold and ∼13-fold in the basal and insulin-stimulated states, respectively. Consequently, the absolute amount of HA-GLUT4 translocated in response to insulin (insulin minus basal cell surface HA-GLUT4) showed only small changes with increasing total cellular expression (Figs. 1 and 3A) whereas the ratio of translocated HA-GLUT4 to total cellular HA-GLUT4 was markedly decreased (Fig. 3C).

3.4. *Effects of coexpression of HA-GLUT4 and FLAG-GLUT4 or wild type GLUT1 in transfected rat adipose cells*

To further investigate the relationship just described, we performed cotransfections with two differently epitope-tagged GLUT4s, HA-tagged and FLAG-tagged GLUT4. This approach was used to monitor the cell surface distribution of the reporter HA-GLUT4 under conditions of both low and high expression levels of total GLUT4. As judged by Western blot analyses of total membranes, adipose cells transfected with either HA-GLUT4 or FLAG-GLUT4 alone showed comparable protein expression levels of the respective tagged GLUT4 (data not shown). In addition, the basal and insulin-stimulated cell surface distributions of HA-GLUT4 and FLAG-GLUT4 were similar (data not shown). Finally, Western blot analyses of total membranes from transfected cells demonstrated that the obtained protein expression levels of HA-GLUT4 were unchanged by coexpression of FLAG-GLUT4 (data not shown).

As shown in Fig. 4A, and above in Fig. 1, in adipose cells transfected with HA-GLUT4 alone, both basal and insulin-stimulated cell surface levels of HA-GLUT4 increased with increasing amounts of HA-GLUT4 expression plasmid. Correspondingly, the observed HA-GLUT4 insulin response decreased with overexpression of the protein, ranging from ∼4.5-fold for 0.1 μg plasmid DNA/cuvette to ∼1.5-fold for 5 μg plasmid DNA/cuvette. However, when cells were cotransfected with expression plasmids for HA-GLUT4 and FLAG-GLUT4, the basal cell surface levels of HA-GLUT4 remained unchanged compared to the control. In contrast, the insulin-stimulated cell surface levels of HA-GLUT4 were dramatically reduced by coexpression of FLAG-GLUT4. Stimulation of these cotransfected cells with insulin resulted in an ∼1.5-fold increase in cell surface HA-GLUT4. Thus, the fold insulin responses of HA-GLUT4 in FLAG-GLUT4, HA-GLUT4-cotransfected cells were similar to the values obtained for cells transfected with saturating amounts of HA-GLUT4 expression plasmid alone (Fig. 4B).

To test whether the targeting of GLUT4 was also affected by overexpression of GLUT1, we cotransfected adipose cells with tracer amounts of HA-GLUT4 plasmid (0.5 μg DNA/cuvette) together with increasing amounts of plasmid for wild type GLUT1. As shown in Fig. 4C, overexpression of GLUT1 did not significantly affect the cell surface distribution of coexpressed HA-GLUT4 in either the basal or insulin-stimulated

states. Likewise, in the reverse experiment, the cell surface levels of tracer HA-GLUT1 were unaffected by coexpression of saturating amounts of FLAG-GLUT4 (data not shown). As judged by Western blotting of total membranes from cells transfected with either HA-GLUT4 or HA-GLUT1 alone with anti-HA antibodies, the obtained protein expression levels of both GLUTs were comparable (data not shown).

4. Discussion

In the present study, we have established the quantitative relationship between the amount of total cellular HA-GLUT4 and HA-GLUT4 in the plasma membrane. In agreement with the findings obtained using transgenic mice [11], basal cell surface levels of HA-GLUT4 in the transfected cells appear roughly proportional to the amount of GLUT4 expressed. In contrast, the insulin-stimulated cell surface HA-GLUT4 levels do not increase proportionally to the expression levels (Fig. 3). Indeed, it appears that the transfected cells fail to target overexpressed GLUT4 to the plasma membrane upon stimulation with insulin rather than failing to retain the excess GLUT4 inside the cell in the absence of hormone. As a result, the total amount of GLUT4 translocated in response to insulin appears to reach a constant value whereas the cellular proportion of HA-GLUT4 in the plasma membrane after insulin stimulation decreases, with increasing expression levels of GLUT4 (Figs. 3 and 4).

This effect is further clearly demonstrated by the coexpression of tracer amounts of HA-tagged GLUT4 together with large amounts of FLAG-tagged GLUT4 (Fig. 4). Coexpression of FLAG-GLUT4 does not lead to changes in basal cell surface levels of tracer HA-GLUT4 as would be expected for saturation of a hypothetical retention mechanism dependent on GLUT4 itself, e.g. a GLUT4 binding protein responsible for intracellular sequestration. In contrast, overexpression of FLAG-GLUT4 leads to a drastic decrease of cell surface HA-GLUT4 in the insulin-stimulated state. The insulin response of tracer HA-GLUT4 in FLAG-GLUT4-cotransfected cells is similar to the insulin response observed in cells transfected with saturating amounts of HA-GLUT4 alone. Thus, these data argue against saturation of a retention mechanism involving GLUT4 itself. Rather, our results indicate that the mechanism for the insulin-stimulated translocation of GLUT4 is saturated by excess GLUT4.

Evidently, the cellular translocation machinery has a limited capacity to recruit excess GLUT4 to the plasma membrane upon insulin stimulation. The molecular mechanisms responsible for the formation and targeting of GLUT4-containing vesicles are not well understood. It is believed that components of the GLUT4-containing vesicles (e.g. soluble NSF attachment protein receptors (SNAREs)) as well as cytosolic proteins (e.g. SNAP-23) participate in the process of vesicle trafficking [20–24]. Thus, a possible explanation for this observed effect might be that such accessory proteins required for the formation of translocation-competent GLUT4 vesicles become limiting when GLUT4 is overexpressed. Likewise, these proteins may be directly involved in the insulin-stimulated translocation of the GLUT4-containing vesicles. This latter concept is supported by the finding that the protein composition of the vesicles containing GLUT4 from adipose cells overexpressing GLUT4 was unchanged except for a marked increase in GLUT4 itself [16]. Further studies in insulin target cells are required to identify the components of the cellular machinery required for the translocation of GLUT4.

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