Dynamics of insulin-stimulated translocation of GLUT4 in single living cells visualised using green fluorescent protein

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Abstract Insulin increases glucose uptake by promoting the translocation of the GLUT4 isoform of glucose transporters to the plasma membrane. We have studied this process in living single cells by fusing green fluorescent protein (GFP) to the N-terminus (GFP·GLUT4) or C-terminus (GLUT4·GFP) of GLUT4. Both chimeras were expressed in a perinuclear compartment of CHO cells, and in a vesicular distribution through the cytosol. Insulin promoted an increase in plasma membrane fluorescence as a result of net translocation of the chimeras to the cell surface. GLUT4·GFP, but not GFP·GLUT4, was re-internalised upon the removal of insulin suggesting that a critical internalisation signal sequence exists in the N-terminus of GLUT4. The use of GFP thus allows an analysis of GLUT4 trafficking in single living cells.

Key words: Glucose transport; Translocation; Insulin; Green fluorescent protein

1. Introduction

Insulin stimulates glucose uptake into adipocytes and muscle cells by virtue of an insulin-regulatable glucose transporter, GLUT4. The translocation of this transporter from an intracellular location to the cell surface in response to insulin is largely responsible for the 20- to 30-fold increase in glucose transport observed in these cells (for review see [1–3]). However, the exact intracellular location of GLUT4 in the basal state and the mechanism by which it reaches the cell surface upon insulin stimulation are unclear. Under basal or non-stimulated conditions more than 95% of the GLUT4 is localised to tubular vesicular elements that are found clustered adjacent to early or late endosomes, in the *trans* Golgi reticulum (TGR) or in the cytoplasm often close to the plasma membrane. The level of GLUT4 is decreased by 40–50% at each of these locations by insulin, suggesting that all of these compartments participate in the insulin regulated recycling of GLUT4 [4,5]. Immuno-electron microscopy (EM) studies of GLUT4 in adipocytes have further shown that GLUT4 is located, at least in part, within the endocytic system as it undergoes constitutive recycling through clathrin-coated pits [5,6].

Several approaches have been used to 'tag' GLUT4 and monitor its subcellular location and/or recycling. These include immunogold electron microscopy [4,5], and radiolabelled membrane-impermeant glucose transporter-specific photolabels that have been developed which irreversibly bind GLUT4 upon exposure to UV light [7–9]. While studies using such 'tags' are highly informative, they are limited by several key factors. Firstly, immuno-EM cannot provide information on the dynamics of the translocation event as cells must be processed, fixed and labelled. Photolabels are limited by their inability to distinguish between the various isoforms of glucose transporter, the multiple intracellular pools of GLUT4, and also the need to solubilise cells and immunoprecipitate GLUT4 to quantitate changes in distribution [10,11]. Indeed, all of these methodologies ultimately rely on cellular disruption prior to analysis. An alternative approach, whereby cell surface glucose transporters can be detected with an epitope tag within the first exofacial loop [12,13], again provides only a static picture of the localisation of the transporter at the cell surface and provides no information on the intracellular distribution. To circumvent these problems, we sought to develop a system which would allow us to monitor GLUT4 translocation in real time in living cells. Such a system will have wide implications for the analysis of GLUT4 trafficking, recycling and the signalling mechanisms involved.

The system involves the use of the *Aequoria victoria* green fluorescent protein (GFP). The expression of this intrinsically fluorescent protein in mammalian cells has generated a considerable amount of interest in the use of this molecule to 'tag' intracellular proteins which can be monitored in situ [14–17]. There are some reports of the successful expression of GFP-fusion proteins in mammalian cells, for example with the NMDA receptor [18], MAP4 [19] and cyclins [20]. However, the extent to which GFP, which is a relatively large (27 kDa) protein, might interfere with the normal regulatable trafficking of a protein in mammalian cells has not been adequately addressed.

We have prepared two chimeras, with GFP fused either to the amino- or carboxyl-terminal ends of GLUT4. Both chimeras exhibit a distribution characteristic of GLUT4 in several cell types. Upon exposure of cells expressing either chimera to insulin, we observed their translocation to the plasma membrane. Of particular interest was the observation that re-internalisation of the transporter upon insulin removal was prevented by fusing GFP to the N-terminus but not C-terminus of GLUT4. The results are discussed in terms of a molecular model for transporter translocation and recycling.

2. Materials and methods

2.1. Materials

Oligonucleotides were synthesised by the University of Bristol Molecular Recognition Centre. Unless otherwise stated all other biochemical reagents were either from Sigma (Poole, Dorset, UK) or BDH (Poole, Dorset, UK). Tissue culture media and foetal calf serum were from Gibco (Paisley, UK). pGFP was generated by subcloning the cDNA encoding the wild-type sequence of GFP from the vector TU65

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Abbreviations: GFP, green fluorescent protein

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(a generous gift of Dr M. Chalfie, Columbia University [14]) into the mammalian expression vector pcDNAneoI (Invitrogen, San Diego, CA). Rabbit polyclonal antibodies raised against a synthetic peptide encoding the C-terminal 14 amino acid residues of the human isoform of GLUT4 have been described previously [21]. Antibody 9E10 reactive against the epitope MEQKLISEEDLK was a gift of H. Patterson [22] (Chester Beatty Laboratories, London).

2.2. Plasmid construction

The plasmids TU65 and pSPGT4 [23] were used as templates for PCR amplification of the wild-type GFP and GLUT4 sequences. This was achieved as follows.

For the N-terminal GFP fusion (GFP·GLUT4), the GFP cDNA was amplified with the sense primer TTTAAGCTTGACGAGATGGAGCAGAAGCTGATCTCGGAGGAGGACCTGAAGAGTAAAGGAGAAGAA (possesses a 5′ *Hin*dIII site followed by a Kozak initiation sequence, initiating methionine, epitope for recognition by antibody 9E10 (underlined) and five codons complimentary to amino acids 2–6 of wild-type GFP) and antisense primer TTTTGGATCCTTTGTATAGTTCATC (possesses a 5′ *Bam*HI site and five codons complimentary to the amino acids 234–238 of GFP). The GLUT4 cDNA was amplified using sense primer TTTTGGATCCCCGTCGGGCTTCCAA (possesses a 5′ *Bam*HI site followed by the first five amino acids of GLUT4 excluding the initiating methionine) and antisense primer TTTTTCTAGATCAGTCGTTCTCATC (possesses a 5′ *Xba*I site followed by the final five amino acids of GLUT4, including the stop codon). The GFP containing PCR product was digested with *Hin*dIII and *Bam*HI, and ligated into the *Hin*dIII–*Bam*HI site of the mammalian expression vector pcDNAneoI. This plasmid was then digested with *Bam*HI and *Xba*I and ligated with the GLUT4 PCR product which had been similarly digested with *Bam*HI and *Xba*I.

For the C-terminal GFP fusion (GLUT4·GFP), the GLUT4 cDNA was amplified with the sense primer TTTTAAGCTTGACGAGATGCCGTCG (possesses a 5′ *Hin*dIII site followed by a Kozak initiation sequence and three codons complimentary to amino acids 1–3 of GLUT4) and antisense primer TTTTGGATCCGTCGTTCTCATCTGG (possesses a 5′ *Bam*HI site and five codons complimentary to amino acids 505–509 of GLUT4). The GFP cDNA was amplified using sense primer TTTTGGATCCAGTAAAGGAGAAGAA (possesses a 5′ *Bam*HI site followed by amino acids 2–6 of GFP) and antisense primer TTTTTCTAGATCACTTCAGGTCCTCCTCCGAGATCAGCTTCTGCTCCATTTTGTATAGTTCATC (possesses a 5′ *Xba*I site followed by the epitope for antibody 9E10 which is underlined, and the final five amino acids of GFP and includes a stop codon). The GFP containing PCR product was digested with *Bam*HI and *Xba*I and ligated into the *Bam*HI–*Xba*I site of pcDNAneoI. The resulting plasmid was then digested with *Hin*dIII and *Bam*HI, and ligated with the GLUT4 PCR product which had been similarly digested with *Hin*dIII–*Bam*HI.

The sequences encoding the final fusion proteins were re-sequenced in their entirety to confirm the absence of spurious mutations, and were purified by banding on two successive CsCl gradients. The resulting plasmid products were resuspended in 2 mM Tris-HCl, 0.2 mM EDTA, pH 8.0.

2.3. Cell microinjection

CHO·T cells, which over-express the human insulin receptor, were seeded onto glass coverslips and were incubated in 60 mm diameter petri dishes in growth medium (Ham's F12 medium supplemented with 5% foetal calf serum, 10 mM Hepes, 200 U·ml^{-1} benzylpenicillin, 100 μg·ml^{-1} streptomycin and 250 μg·ml^{-1} G418) and cultured at 37°C in an atmosphere of 5% CO_2 until approx. 80% confluent. The plasmids containing the chimeras (200 μg/ml, in 2 mM Tris-HCl, pH 8.0, 0.2 mM EDTA) were microinjected into cells in DMEM supplemented with 5% foetal calf serum, 25 mM Hepes, 200 U·ml^{-1} benzylpenicillin, 100 μg·ml^{-1} streptomycin and 2 mM $NaHCO_3$. Cells were then incubated in growth medium for 16–24 h prior to experiments.

2.4. Fluorescence analysis

Microinjected cells were serum starved for 2 h prior to fluorescence analysis in Hepes-buffered Krebs (10 mM Hepes, pH 7.4, 2 mM $NaHCO_3$, 140 mM NaCl, 3.6 mM KCl, 0.6 mM Na_2HPO_4, 0.5 mM $MgSO_4$, 1 mM $CaCl_2$ and 5.5 mM glucose) using a Zeiss Axiovert 100TV microscope with a 40× oil-immersion objective, and a cooled charge couple device camera (Photonic Science, UK) with BioVision image analysis software (Improvision, University of Warwick Science Park, UK). GFP exitation/emission was achieved with a High Q FITC filter set (41001; Chroma Technology Corp. Brattleboro, VT). If required, the cells were then further incubated as described in the figure legends and re-imaged.

For immunofluorescence analysis, the cells were fixed in 4% paraformaldehyde in PBS for 20 min and permeabilised with 0.015% saponin in PBS for 10 min. This, and subsequent steps, were performed at room temperature. The cells were blocked with 3% bovine serum albumin in PBS (BSA/PBS) for 15 min and then incubated in BSA/PBS containing 1:100 dilution of anti-Myc antibody 9E10 [22] for 1 h. Cells were washed 3 times for 5 min in PBS and incubated in 1:100 dilution of a rhodamine-conjugated goat anti-mouse IgG (Dako, Denmark) in BSA/PBS for 1 h. After extensive washing in PBS the coverslips were mounted in Mowiol and fluorescence imaging performed as described above but with Zeiss filter set 14 for excitation/emission.

3. Results

Two GLUT4 fusion proteins were generated which contained the entire coding sequence of GLUT4 with GFP fused either at the N- or C-terminus. For the sake of simplicity we will refer to the fusion constructs as pGFP·GLUT4 and pGLUT4·GFP, respectively. An epitope tag was engineered into the fusion proteins either at the very N-terminus of GFP·GLUT4, or C-terminus of GLUT4·GFP. This epitope is based on the amino sequence recognised by the monoclonal anti-Myc antibody 9E10, and was incorporated to allow additional analysis by immunofluorescence staining of fixed and permeabilised cells.

3.1. Expression of GFP·GLUT4 and GLUT4·GFP in CHO·T cells

We used CHO cells that over-express human insulin receptors (CHO·T) because they allow the greatest level of GFP fluorescence. In our hands, GFP exhibits considerably lower levels of fluorescence in other types of fibroblast (e.g. 3T3-L1), and no detectable fluorescence in PC12 cells (S.D. and J.M.T., unpublished data). It is not clear why GFP exhibits such a wide variety of different levels of fluorescence intensity; it may be related to the redox state of the cell, small variations in intracellular pH, differences in oxygen tension or expression of chaperonins. Whatever the reason, the experiments described below were only possible in the CHO cell background.

When native GFP is expressed in CHO·T cells it exhibits a diffuse distribution throughout the cell but with a slight concentration in the nucleus and exclusion from nucleoli (Fig. 1a). By contrast, fluorescence was detected in the perinuclear region of cells expressing either the GLUT4·GFP (Fig. 1b) or GFP·GLUT4 (Fig. 1c) chimeras. A punctate and vesicular-like distribution was also observed throughout the cytosol (Fig. 1b,c and see also below). Both chimeras were excluded from the nucleus, and the plasma membrane was devoid of any significant fluorescence. That the chimeras were excluded from the nucleus strongly suggests that they were intact, and that the junction between the two proteins was not susceptible to proteolysis to generate native GFP. Consistent with this, we found that GFP·GLUT4 over-expressed in COS cells exhibited an apparent M_r of approx. 70–90 kDa (data not shown). This represented a shift in M_r of approx. 20–40 kDa when compared to native GLUT4, and was the expected increase in molecular weight of the chimera considering the molecular mass of GFP is 27 kDa.

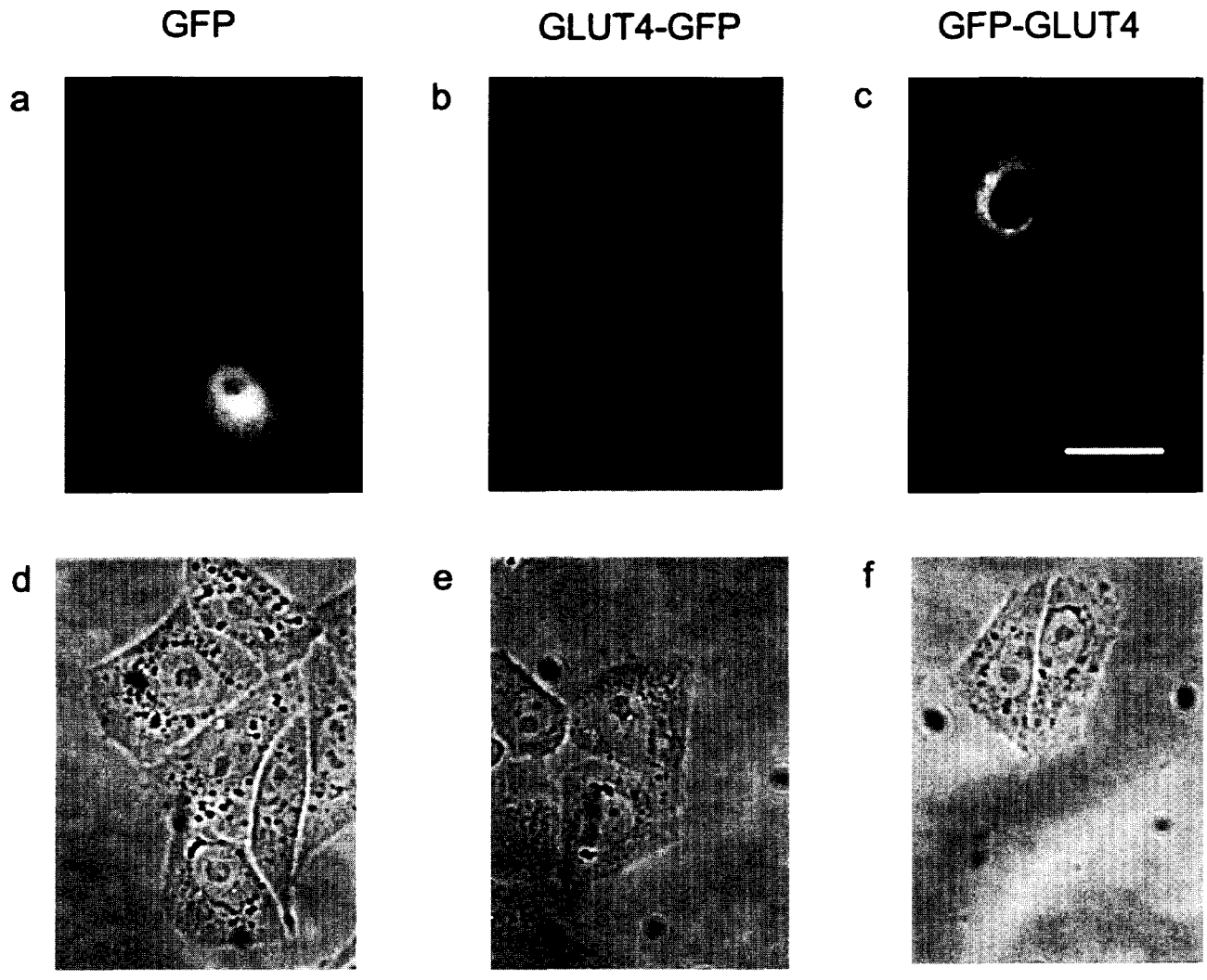

Fig. 1. Expression of GFP·GLUT4 and GLUT4·GFP in CHO·T cells: comparison with native GFP. CHO·T cells were microinjected with pGFP (a,d), pGLUT4·GFP (b,e) or pGFP·GLUT4 (c,f) cDNAs. The cells were incubated in complete medium for 24 h before taking a fluoresence (a–c), or bright-field (d–f) images. The bar represents 10 μm.

In some experiments a very bright and localised region of fluoresence was also observed close to the nucleus and overlapping with the perinuclear distribution described above (see Fig. 2a–d). This bright, almost circular area of fluoresence, suggests some expression at, or close to, the microtubule organising centre.

We noted that if the cells were incubated for longer than 48 h after microinjection, the intensity of plasma membrane fluorescence increased dramatically (data not shown). This suggests that as the expression level of the chimeras increases beyond a threshold level, the chimeras may leak out of the intracellular compartment and into the plasma membrane. Thus, the intracellular compartment can only retain a finite amount of chimeric transporter. All subsequent experiments were thus performed within 24 h of microinjection.

3.2. *Insulin stimulates translocation of both GLUT4 chimeras to the plasma membrane*

We next assessed the effect of insulin on the translocation of the chimeras to the plasma membrane. Cells expressing either GLUT4·GFP or GFP·GLUT4 were imaged prior to treatment with insulin. As shown in the top set of panels in Fig. 2, the distribution of the transporters in the basal state was similar to that shown in Fig. 1. The two cells shown in Fig. 2a,b exhibited an increased plasma membrane fluorescence after treatment with insulin (e and f, respectively). Very similar results were obtained with the GFP·GLUT4 chimera (compare c and d with g and h, respectively).

In the absence of insulin, we consistently observed a proportion of cells (20 ± 5%; mean ± SEM of three separate experiments) that exhibited pronounced plasma membrane-localised GLUT4 chimeras. The addition of insulin for 1 h increased this proportion of cells to 49 ± 3%. Thus approximately half of the cells did not respond to insulin as judged by this criterion. However, the non-responsive cells did express insulin receptors as they immunostained with a monoclonal anti-insulin receptor antibody (data not shown).

To confirm that insulin promoted a net translocation of the GLUT4 chimeras to the plasma membrane, we stained fixed and permeabilised cells expressing GFP·GLUT4 with the monoclonal antibody 9E10 which recognises the short peptide epitope inserted at the N-terminus of the molecule. As shown

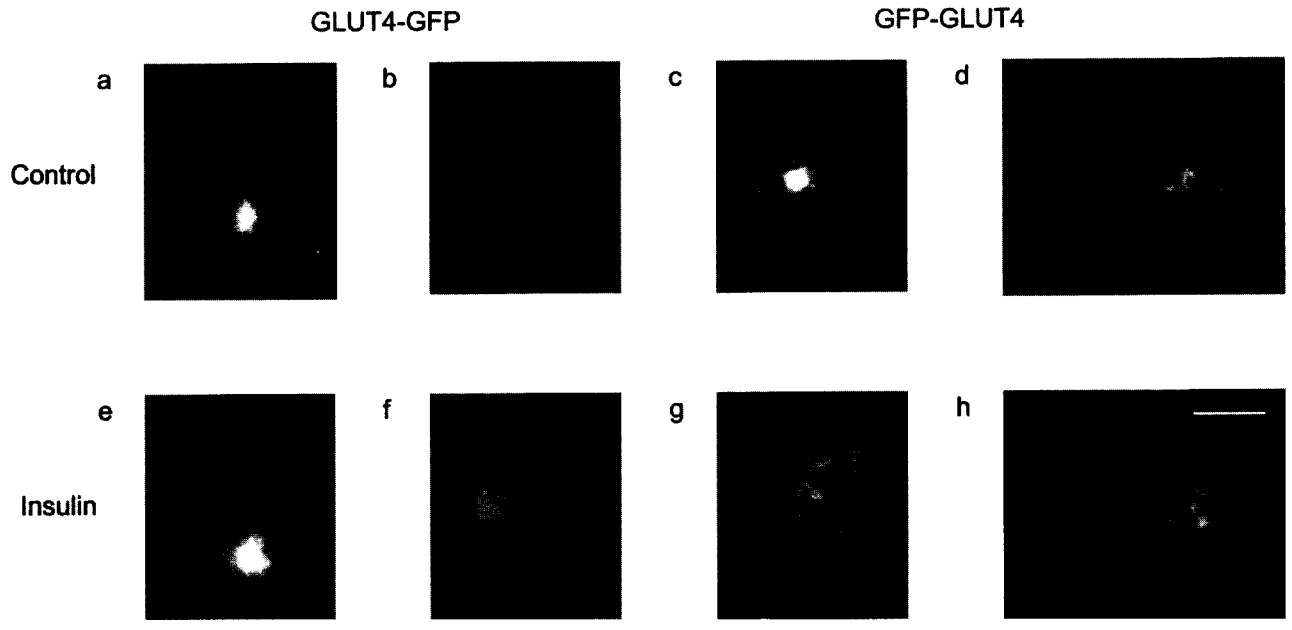

Fig. 2. Effect of insulin on the subcellular distribution of GFP·GLUT4 and GLUT4·GFP chimeras in living CHO·T cells. CHO·T cells were microinjected with pGLUT4·GFP (a,b,e,f) or pGFP·GLUT4 (c,d,g,h) and incubated in complete medium for 16 h. The cells were incubated in serum-free Ham's F12 medium for 2 h, and a basal fluorescence image then taken in Krebs medium (a–d). The cells were incubated in serum-free Ham's F12 medium containing 200 nM insulin and a second fluorescence image of the same cells in panels a–d were taken 1 h later in Krebs medium (e–h, respectively). The bar represents 10 μm.

in Fig. 3, insulin treatment of the cells did indeed promote an increase in immunoreactive chimera at the plasma membrane.

3.3. Endocytosis of GLUT4 chimeras upon insulin removal

We next examined the ability of the chimeras to re-internalise upon subsequent removal of insulin. To do this, we treated cells expressing GLUT4·GFP or GFP·GLUT4 with insulin for 1 h at 37°C. Fluorescence analysis of cells confirmed translocation of each chimera to the plasma membrane (Fig. 4a–d). Insulin was removed, and the cells further incubated overnight at 20°C. This lower temperature was used to prevent the build up in expression that causes the chimeras to leak out to the plasma membrane after 48 h at 37°C (see discussion above).

The GLUT4·GFP chimera exhibited almost complete re-internalisation upon insulin removal (compare Fig. 4a with e, and b with f). When these cells were subsequently re-challenged with insulin for 1 h at 37°C, the GLUT4·GFP chimera returned to the plasma membrane demonstrating that the effect was fully reversible (data not shown). In contrast, we observed no detectable re-internalisation of the GFP·GLUT4 chimera (compare Fig. 4c with g, and d with h).

4. Discussion

The cloning of GFP, and the ability to express this molecule in a variety of heterologous systems and organisms, has provided a particularly useful tool for introducing a fluorescent 'tag' into proteins and following their intracellular trafficking in living cells. A number of properties of GFP lend itself for this purpose including the intrinsic formation of the fluorophore in the absence of added co-factor and its monomeric nature [17]. On the other hand its relatively large molecular mass (27 kDa) compared to chemical fluorophores, could promote mis-targeting of the fusion protein, and cause abnormal trafficking or sequestration as a result of steric effects.

In fusing GFP to either the N- or C-terminus of the insulin-responsive glucose transporter, GLUT4, we found that the fusion proteins were expressed in a perinuclear location of CHO cells and vesicles spread throughout the cytosol, but were excluded from the plasma membrane (Figs. 1 and 2). This is an expression pattern which is very similar, at least

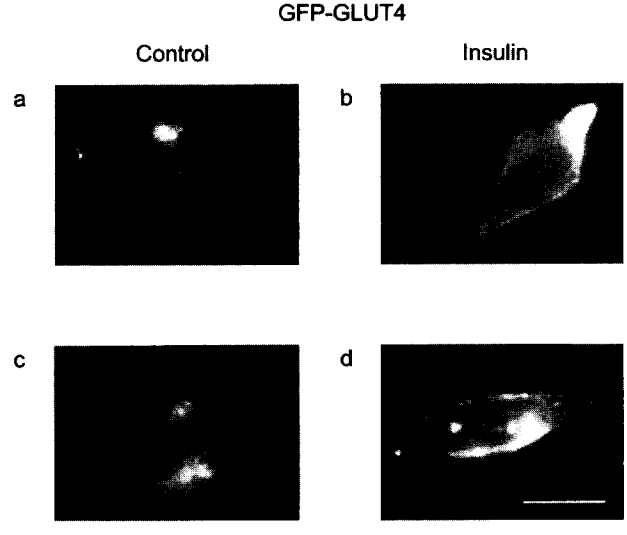

Fig. 3. Immunofluorescence analysis of GFP·GLUT4 expressed in CHO·T cells. CHO·T cells were microinjected with pGFP·GLUT4 and incubated in complete medium for 16 h. The cells were serum starved for 2 h, and then incubated for 1 h in the absence (a,c) or presence (b,d) of 200 nM insulin for 1 h. The cells were fixed, permeabilised and stained with antibody 9E10 as described in Section 2. The bar represents 10 μm.

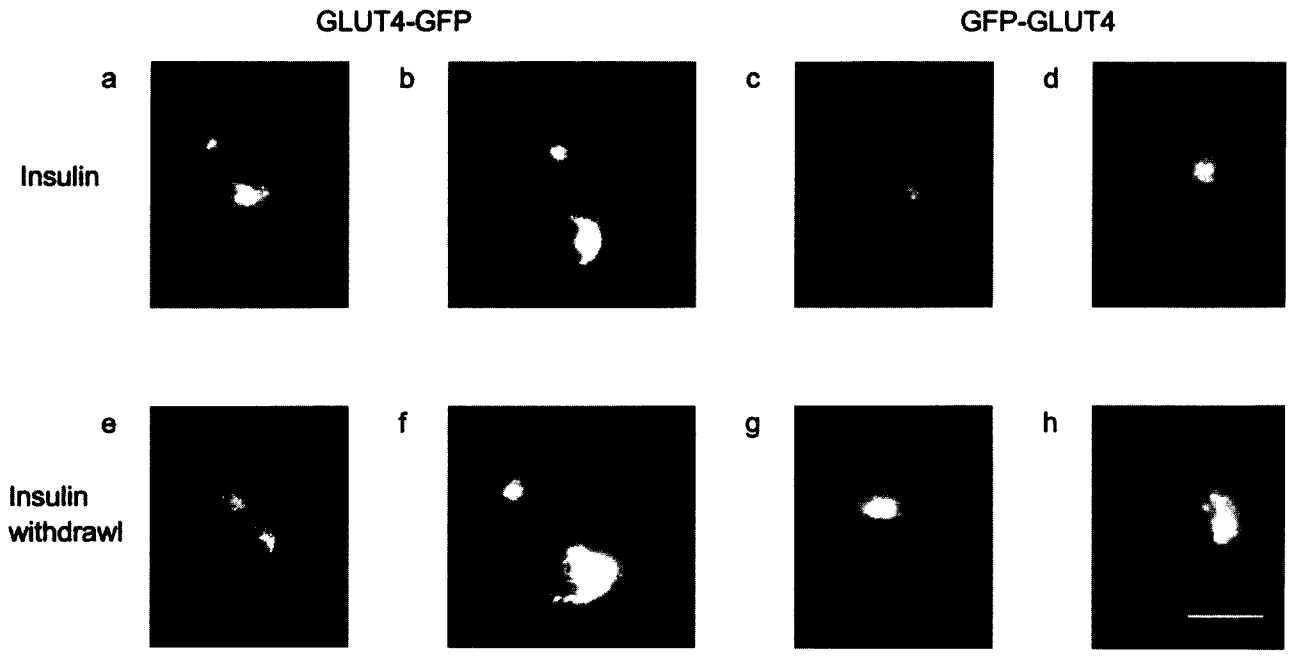

Fig. 4. Internalisation of GLUT4·GFP and GFP·GLUT4 chimeras upon insulin removal. CHO·T cells were microinjected with pGLUT4·GFP (a,b,e,f) or pGFP·GLUT4 (c,d,g,h) and incubated in complete medium for 16 h. The cells were incubated in serum-free Ham's F12 medium for 2 h, followed by 200 nM insulin for 1 h. A fluorescence image was taken in Krebs medium (a–d). The cells were washed thoroughly in serum-free DMEM followed by continued incubation in the absence of insulin in serum-free Ham's F12 medium for a further 16 h at 20°C. The same cells were then re-imaged (e–h). The bar represents 10 μm.

by fluorescence microscopy, to heterologously expressed native GLUT4 in CHO cells [24,25], PC12 cells [26] and 3T3-L1 cells [27,28], or endogenous GLUT4 in 3T3-L1 cells [27].

Previous studies have concluded that GLUT4 possesses at least two signals that may be responsible for targeting the transporter to this intracellular location in fibroblasts. One, a phenylalanine-based (FQQI) motif, is close to the N-terminus [25,27] and the other, a dileucine motif, is found in the C-terminal tail [29,30]; however, other motifs may be involved [13]. Mutagenesis of either motif causes GLUT4 to be targeted to the plasma membrane of cells (reviewed in [3]). The close apposition of GFP to either of these motifs did not appear to prevent intracellular sequestration (Figs. 1 and 2) suggesting either that GFP does not hinder the recognition of these motifs by the sorting mechanism, or that other intracellular sequestration motifs exist on GLUT4 in any one of the five intracellular loops of the structure. The latter would be consistent with experiments of Ishii et al. [13] who found that the middle intracellular loop of GLUT4 provided an additional sequestration signal in CHO cells.

Treatment of cells with insulin promoted an increase in plasma membrane fluorescence (Fig. 2). Using immunofluorescence analysis of fixed and permeabilised cells with an antibody that recognised an epitope tag present only within the chimeras, we demonstrated this to be the result of net translocation of the chimera to the cell surface (Fig. 3). Both chimeras appeared to respond to a similar extent and thus, again, GFP does not appear to hinder the translocation machinery. This is, perhaps, not surprising as other proteins (e.g. synaptobrevins and/or SCAMPs) within the GLUT4 containing vesicle are likely to be involved in this process [31,32], and GLUT4 itself plays only a passive role.

Of particular interest was the difference in internalisation characteristics of the two chimeras. The data shown in Fig. 4 demonstrate that fusion of GFP at the N-terminus of GLUT4 effectively blocked re-internalisation of GLUT4 when insulin was removed from the medium. This was in contrast to GLUT4 with GFP fused at the C-terminus which exhibited an almost compete re-internalisation into a vesicular compartment (Fig. 4). This data suggests that the major internalisation motif is present at the N-terminus of GLUT4, and that its interaction with the internalisation machinery is sterically hindered by the presence of a large bulky GFP molecule. Studies with a chimeric transferrin receptor possessing a cytoplasmic tail derived from the N-terminus of GLUT4 suggest that this domain of GLUT4 is involved in efficient internalisation and not subcellular localisation of GLUT4 in CHO cells [33]. Our data now suggest that the N-terminal domain of *full-length* GLUT4 possesses a critical internalisation motif. This motif is likely to be the phenylalanine-based (FQQI) sequence which is important for GLUT4 sequestration in CHO cells [25] and 3T3-L1 cells [27]. However, we cannot completely exclude the possibility that N-terminal GFP also sterically hinders the C-terminal dileucine motif; however, this is rather unlikely in view of the fact that GFP placed at the C-terminus of GLUT4 does not hinder internalisation.

Our results with the GFP·GLUT4 chimera also suggest that once synthesised in the endoplasmic reticulum, the chimeric transporters are sorted directly into the intracellular compartment, rather than being exported first to the plasma membrane and then internalised to the intracellular compartment. If the latter were the case, then GFP·GLUT4 would have exhibited pronounced plasma membrane localisation in the absence of insulin due to its inability to be internalised; this was not the case (Figs. 1c and 2c,d).

Our single cell approach to studying insulin action has begun to reveal a particularly intriguing heterogeneous response of the cells to insulin. We always observe a proportion of cells

(≈50%) that do not respond to insulin during the course of an experiment. We have previously developed an assay for insulin-stimulated gene transcription in single living cells [34] which was based on the detection of luminescence from CHO-T cells microinjected with a plasmid possessing the firefly luciferase gene under the control of an insulin-responsive promoter. We have also observed a similar heterogeneity with respect to the induction of luciferase expression in individual cells. Some cells respond rapidly to insulin, others more slowly, and some do not respond at all [34]. While the reasons for this heterogeneity are not known, we have previously proposed that it may be due to the point at which the cell is within the cell cycle. For example, a protein kinase activity may be up-regulated during one phase of the cell cycle that inhibits one of the components of the insulin signalling pathway. It will be of particular interest to determine whether cells that exhibit a lack of insulin-stimulated GLUT4 trafficking, also exhibit a reduced activation of gene transcription.

In conclusion, therefore, we have demonstrated that GLUT4 trafficking can be followed in living cells by generating fusion proteins with GFP attached either at the N- or C-terminus of this transporter. Analysis of these chimeras in CHO cells suggests a critical role for the GLUT4 N-terminus in GLUT4 internalisation from the plasma membrane.

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