Identification and characterization of two distinct intracellular GLUT4 pools in rat skeletal muscle: evidence for an endosomal and an insulin-sensitive GLUT4 compartment

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In skeletal muscle, acute insulin treatment results in the recruitment of the GLUT4 glucose transporter from intracellular vesicular structures to the plasma membrane. The precise nature of these intracellular GLUT4 stores has, however, remained poorly defined. Using an established skeletal-muscle fractionation procedure we present evidence for the existence of two distinct intracellular GLUT4 compartments. We have shown that after fractionation of crude muscle membranes on a discontinuous sucrose gradient the majority of the GLUT4 immunoreactivity was largely present in two sucrose fractions (30 and 35%, w/w, sucrose; denoted F30 and F35 respectively) containing intracellular membranes of different buoyant densities. Here we show that these fractions contained 44 ± 6 and 49 ± 7% of the crude membrane GLUT4 reactivity respectively, and could be further discriminated on the basis of their immunoreactivity against specific subcellular antigen markers. Membranes from the F30 fraction were highly enriched in transferrin receptor (TfR) and annexin II, two markers of the early endosome compartment, whereas they were significantly depleted of both GLUT1 and the α1-subunit of $(Na^+ + K^+)$-ATPase, two cell-surface markers. Insulin treatment resulted in a significant reduction in GLUT4 content in membranes from the F35 fraction, whereas the amount of GLUT4 in the less dense (F30) fraction remained unaffected by insulin. Immunoprecipitation of GLUT4-containing vesicles from both intracellular fractions revealed that TfR was present in GLUT4 vesicles isolated from membranes from the F30 fraction. In contrast, GLUT4 vesicles from the F35 fraction were devoid of TfR. The aminopeptidase, vp165, was present in GLUT4 vesicles from both F30 and F35; however, vesicles isolated from F30 contained over twice as much vp165 per unit of GLUT4 than those isolated from F35. The biochemical co-localization of vp165/GLUT4 was further substantiated by double-immunogold labelling of ultrathin muscle sections. Overall, our data indicate the presence of at least two internal GLUT4 pools: one possibly derived from an endosomal recycling compartment, and the other representing a specialized insulin-sensitive GLUT4 storage pool. Both pools contain vp165.

INTRODUCTION

One of the primary functions of insulin is to facilitate the disposal of blood glucose into peripheral tissues during the post-absorptive state [1]. The principal means by which blood glucose is lowered involves the activation of glucose transport in insulin target cells such as adipocytes and myocytes [2,3]. This activation relies on recruitment of the insulin-responsive GLUT4 glucose transporter from an intracellular storage compartment to the plasma membrane. The increased GLUT4 copy number in the plasma membrane results in an increased rate of glucose uptake which, in skeletal muscle for example, may be elevated by up to fivefold over that observed in the absence of insulin [4].

The provision of GLUT4 molecules from the intracellular compartment to the plasma membrane is an integral part of the mechanism by which insulin stimulates glucose transport in both fat and skeletal muscle. However, we know very little about the cellular mechanisms involved in GLUT4 sorting or of the nature of the intracellular GLUT4 pool. Currently, there are two ways of viewing how the insulin-stimulated translocation of GLUT4 to the plasma membrane from the intracellular pool may take place. One view is that insulin-stimulated GLUT4 translocation resembles regulated secretion, a process in which specialized exocytic vesicles fuse with the plasma membrane [5,6]. A characteristic hallmark of regulated exocytosis is that the exocytic vesicles are held in stasis and are distinct from membrane vesicles participating in the constitutive recycling pathway. Vesicles held in stasis may therefore be considered as specialized membranous structures or organelles which contain a distinct set of proteins enabling the vesicles to be targeted and allowing them to undergo regulated fusion with the cell surface [5,6]. An alternative view suggests that a 'regulated recycling' model is more appropriate than the 'regulated exocytic' model [7–9]. James and colleagues [10] have proposed a consensus model. In the absence of insulin, a fraction of the total internal GLUT4 could be recycling constitutively at the cell surface, while the remaining fraction could be efficiently retained in a specialized storage compartment. On insulin binding, the specific intracellular retention of GLUT4 is relieved and this induces its discharge into the recycling pathway. Insulin is also proposed to accelerate the rate of recycling, and this would result in a specific increase in GLUT4 abundance at the cell surface and thus an increase in glucose transport.

Although speculative, the consensus model provides a useful framework and incorporates many of the apparently contradictory ideas and observations previously reported in the literature. Moreover, the basic concept of two intracellular GLUT4 compartments is in broad agreement with the findings of Holman's group [11], who have suggested, on the basis of

Abbreviation used: TfR, transferrin receptor.

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mathematical analyses and kinetic modelling, that two, rather than just one, intracellular GLUT4 pools best describe the observed rate of activation of glucose transport and GLUT4 recycling in the presence of insulin. Here we report the isolation of a previously uncharacterized GLUT4-containing vesicle pool which is endowed with the transferrin receptor (TfR), a marker of the recycling pathway in eukaryotic cells. In addition, a second insulin-sensitive GLUT4 vesicle pool, lacking TfR, is also present, which may represent the storage pool postulated by the consensus model. We speculate that, in accord with this model, these GLUT4 pools are likely to play different physiological roles in the trafficking of GLUT4 within the skeletal-muscle cell. The biochemical isolation of these two compartments, reported in this study, should prove useful in the further experimental testing of some of the proposed attributes of the consensus model.

MATERIALS AND METHODS

Animals and experimental procedures

Male Sprague–Dawley rats (250 g) were used throughout (Bantin & Kingman, Hull, U.K.). Animals were deprived of food overnight and placed under terminal anaesthesia using a combination of Hipnorm (fentanyl citrate, 0.2 mg/kg; fluanisone, 7 mg/kg; intraperitoneal injection) and Hipnoval (a muscle relaxant, midazolam hydrochloride, 1.5 mg/kg; intraperitoneal injection) before use. In some experiments, animals were separated into two groups. One group received a supramaximal intravenous dose of human insulin (1.5 units) prepared in 0.9 % saline solution, and the second group was given saline alone. At 30 min after administration of insulin or saline, animals were killed, and hindlimb muscles were rapidly excised, frozen in liquid N_2 and subsequently stored at -80 °C until required for study.

Subcellular fractionation of rat skeletal muscle

The procedure for isolating crude muscle membranes from rat skeletal muscle has previously been reported in detail [12]. Briefly, 10–15 g of skeletal-muscle tissue was isolated from rat hindlimbs, homogenized, and subjected to a series of differential centrifugation steps to obtain crude muscle membranes. In initial experiments (shown in Figure 1) these membranes (2–4 mg of protein) were applied to a 10–40 % (w/w) continuous sucrose gradient and spun for 16 h at 190000 ***g***. For preparative purposes, all subsequent fractionation experiments were carried out using 30 mg of crude muscle membrane protein which was fractionated on a discontinuous (25, 30 and 35 %, w/w) sucrose gradient as described previously [12,13].

PAGE and Western-blot analysis

Muscle membrane fractions were subjected to SDS/PAGE, as described by Laemmli [14], on either 7 or 9 % resolving gels. Separated proteins were electrophoretically transferred to nitrocellulose sheets, blocked with 3 % BSA in tris-buffered saline and incubated with polyclonal antibodies to GLUT4 (1:500), GLUT1 (1:1000) (East Acres Biologicals), annexin II (1:400) [15] (a gift from Professor V. Gerke, Clinical Research Group for Endothelial Cell Biology, University of Münster, Münster, Germany) or the aminopeptidase vp165 at 5 μg/ml [16] or monoclonal antibodies to the transferrin receptor (1:1000) (Hybrid Tech), α1-subunit of dihydropyridine receptor (1:50000) (Upstate Biotechnology) and the α1- and α2-subunits of the $(Na^+ + K^+)$-ATPase both at 1:100 dilution [17] (kindly provided by Dr. K. Sweadner, Laboratory of Membrane Biology, Harvard Medical School, Massachusetts General Hospital, Boston, MA, U.S.A.). After antibody incubation, membranes were washed with 0.05 % Tween 20 in TBS before incubation with 0.1 μCi/ml ^{125}I-Protein A for the detection of polyclonal antibodies or ^{125}I-labelled anti-mouse IgG for the detection of monoclonal antibodies. Membranes were subsequently washed and air-dried, and autoradiography was performed by either electronic imaging using a Canberra Packard InstantImager or exposure to XAR-5 Kodak film at -80 °C. vp165 (and in some cases GLUT4) was detected using a horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence. Autoradiographs were quantified using a Bio-Rad GS-670 imaging densitometer.

Immunoisolation of intracellular GLUT4-containing vesicles

The procedure for immunoisolating GLUT4-containing vesicles from muscle has previously been described in detail [18,19]. Briefly, 200 μg of membrane protein recovered from the top of the 30 % (F30) and 35 % (F35) sucrose bands (representing membranes of intracellular origin, see below), were treated with 5 μl of non-immune serum or 5 μl of anti-GLUT4 serum. The GLUT4-containing vesicles were complexed by the addition of Protein A–Sepharose beads and pelleted by centrifugation. The resulting supernatant was centrifuged at 190000 ***g***; the pellet from this step was washed twice with PBS and then resuspended in Laemmli buffer and termed the immune supernatant. The immune pellet was washed four times with PBS before resuspension in Laemmli buffer. During the course of this work an alternative protocol was also established which allowed us to improve the overall efficiency with which GLUT4 vesicles were precipitated. In this protocol, purified anti-GLUT4 antibody (Genzyme Diagnostics, Cambridge, MA, U.S.A.) as well as non-specific mouse IgG (Sigma) were coupled to Protein G–Sepharose beads at a concentration of 1 mg/ml. The antibodies were then cross-linked to the beads using dimethyl pimilimidate. Cross-linked anti-GLUT4 or non-specific mouse IgG (20 μg) incubated with 200–300 μg of membrane protein from F30 and F35 for 1 h at room temperature. Samples were then applied to a discontinuous sucrose gradient (10 and 40 %, w/w, sucrose) and centrifuged at 190000 ***g*** for 30 min. The membranes recovered from the top of the 40 % sucrose band were diluted with PBS and pelleted at 190000 ***g***. The pellet from this step was resuspended in Laemmli buffer and termed the immune supernatant. The beads and vesicles bound to them were collected from the bottom of the sucrose gradient tube and washed twice with PBS before a single wash with 50 mM Hepes, pH 7.4, containing 1 M NaCl and 1 mM EDTA, followed by two final washes in PBS. The adsorbed material was eluted with Laemmli buffer.

Double-labelling immunoelectron microscopy

Food-deprived and anaesthetized rats were prepared for perfusion with 2 % paraformaldehyde in PBS as previously described [20]. Soleus muscle was excised, fixed by immersion in 2 % paraformaldehyde in PBS for 2 h, infiltrated with 2.3 M sucrose in 0.1 M phosphate buffer, pH 7.2, and processed for ultra-cryosectioning as described previously [20]. Ultrathin cryosections were first blocked with 0.5 % BSA and 0.15 % glycine in PBS before incubation with anti-GLUT4 1F8 monoclonal antibody (used at 1.48 μg/ml in PBS/0.5 % BSA). Sections were washed with 0.5 % BSA in PBS (four washes of 5 min) and then incubated for 1 h with 5 mn gold-labelled goat anti-mouse IgG (Amersham Canada, Oakville, Ontario, Canada). Sections were then washed in PBS (3×5 min) and fixed in 2 % paraformaldehyde for 5 min before three further 5 min washes with

PBS. The reactive aldehydes were blocked with 80 mM NH_4Cl (2×5 min) and grids further processed for labelling with anti-vp165 polyclonal antibody (48 μg/ml in 2% Carnation powdered non-fat milk in PBS) and 10 nm gold-labelled goat anti-rabbit IgG (Amersham Canada). Finally, grids were subjected to 6×5 min washes in PBS and a further 4×5 min wash with distilled water before staining and stabilizing with 0.2% uranyl acetate and 2% methylcellulose. Controls included (i) the use of non-immune serum and (ii) PBS instead of primary antibodies. In addition, single-labelling control experiments were conducted in the following combinations: (i) 1F8/5 nm gold goat anti-mouse IgG and PBS/10 nm gold goat anti-rabbit IgG, (ii) PBS/5 nm gold goat anti-mouse IgG and vp165/10 nm gold goat anti-rabbit IgG, (iii) 1F8/5 nm gold goat anti-mouse IgG alone and (iv) vp165/10 nm gold goat anti-rabbit IgG alone to verify that double labelling did not interfere with the recognition of each epitope. Specimens were observed through a Phillips EM 401 transmission electron microscope.

RESULTS AND DISCUSSION

Presence of GLUT4 in membranes of different buoyant density

One biophysical parameter that can be used to characterize biological membranes is their buoyant density. We thought that if a sample of crude membranes housed more than one intracellular pool of GLUT4 glucose transporters, as postulated in our working hypothesis, we could exploit potential differences in membrane density to segregate these pools. Crude muscle membranes were fractionated by application to a continuous (10–40%) sucrose gradient. Figure 1 shows the GLUT4 immunoreactivity profile observed in fractions collected sequentially from a continuous sucrose density gradient. The fractionation procedure clearly led to the resolution of two major immunoreactive GLUT4 peaks. One of these peaks had a rather narrow density distribution around 1.157 g/ml, and the other originated from membrane fractions showing a wider density distribution

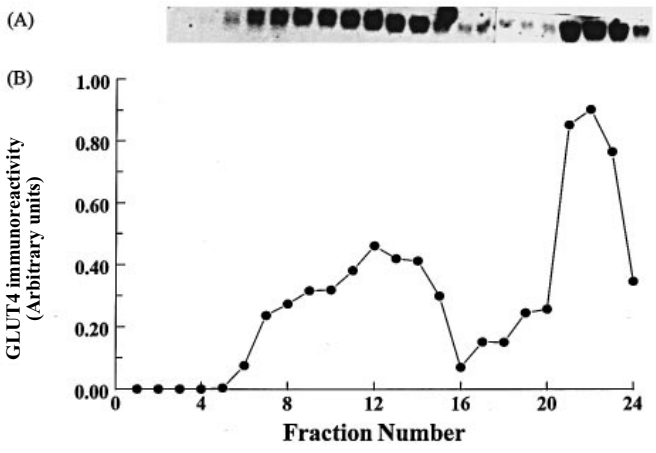

Figure 1 Fractionation of rat muscle crude membranes in a continuous sucrose-density gradient

Crude membranes from rat skeletal muscle were fractionated by applying 2–4 mg of protein to a 10–40% continuous sucrose gradient and spinning for 16 h at 190000 ***g***. Fractions were collected and subjected to SDS/PAGE and immunoblotting using an antibody to GLUT4. (**A**) GLUT4 immunoreactivity in odd-numbered fractions collected from the sucrose gradient. The density of the fractions increases from left to right. (**B**) Quantitative analysis of GLUT4 distribution throughout the gradient. GLUT4 immunoreactivity was quantified and plotted as arbitrary densitometric units.

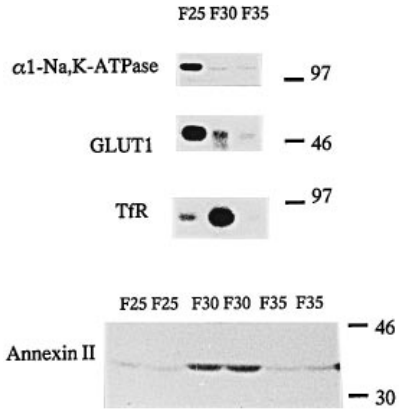

Figure 2 Plasma-membrane and endosomal antigen-marker distribution in discontinuous sucrose-gradient fractions

Muscle membrane samples obtained from a discontinuous sucrose-density gradient as described in the Material and methods section were analysed by SDS/PAGE and Western blotting for the presence of GLUT1, α1-subunit of the $(Na^+ + K^+)$-ATPase, TfR and annexin II. The blot for annexin II depicts data from two separate muscle preparations run side by side. In order to highlight potential differences in protein expression between muscle fractions, the amount of protein loaded on to the gels was kept low (20 μg) and film exposures kept as short as possible (< 10 h) to ensure that signal intensities were within the linear range of the detection method.

ranging between 1.085 and 1.135 g/ml. One possible explanation for the broad plateau distribution could be the presence of distinct GLUT4-containing membrane fractions which have overlapping densities. This suggestion is in line with the observation that, when a discontinuous sucrose gradient (25, 30 and 35% w/w, sucrose) is used, the membranes comprising the broad GLUT4 peak in the continuous sucrose gradient can be separated into two fractions floating on top of the 25% (F25) and 30% (F30) sucrose cushions, which possess a significant cytochalasin B-binding component [21]. Previous enzymic and immunological data indicate that membranes from F25 and F35 are enriched with plasma membranes and intracellular membranes respectively [21–23]. In contrast, no extensive characterization of the membranes recovered from F30 has been performed. Given that these membranes house a significant amount of GLUT4, we have, using antibodies to specific marker proteins, attempted to characterize these membranes further. Data from both immunocytochemical and immunoblotting studies indicate that, in skeletal muscle, expression of the GLUT1 glucose transporter and $(Na^+ + K^+)$-ATPase α1 subunit is restricted to the plasma membrane [13,24]. Consistent with previous findings, both these proteins were predominantly detected in membranes from F25, their reactivity in membranes isolated from F30 and F35 being significantly less (Figure 2). Interestingly, however, when membranes from the discontinuous sucrose gradient were screened with an antibody to TfR, which is generally accepted as being a good marker of the recycling endosomal/plasma-membrane compartment [25], TfR reactivity was largely confined to membranes from F30. Substantially less TfR was detected in membranes from F25 and F35 (Figure 2). Annexin II, a Ca^{2+}- and phospholipid-binding protein that has been implicated in endosomal membrane function [15,25] and which has previously been shown to be expressed in skeletal muscle [26], was also largely confined to membranes from F30 (Figure 2). The observed enrichment of TfR and annexin II in F30 would imply that membranes present in this fraction are largely of endosomal origin.

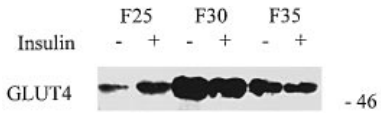

Figure 3 Effects of insulin on the subcellular distribution of GLUT4 in muscle membrane fractions

A representative Western blot is given showing the subcellular distribution of GLUT4 in F25, F30 and F35 fractions (25 μg of protein each) prepared from control (−) and insulin-treated (+) skeletal muscle as described in the Materials and methods section.

Insulin sensitivity of the GLUT4 pools found in F30 and F35 fractions

We next investigated the effect of acute *in vivo* insulin treatment on the redistribution of GLUT4 in these fractions. To this end, we immunoblotted membranes from the F25, F30 and F35 fractions prepared from control and insulin-treated rat skeletal muscle. Figure 3 depicts a representative Western blot showing the effects of insulin on the subcellular distribution of GLUT4. Quantitative densitometry of three muscle preparations revealed that acute insulin treatment caused a 1.9 ± 0.5 fold increase in GLUT4 reactivity in the F25 (plasma membrane) fraction. We were unable to observe any detectable changes in GLUT4 abundance in the F30 fraction after insulin treatment. Scanning analysis of data from three independent experiments showed that the intensity of the GLUT4 signal in the F30 fraction from insulin-treated rats was 1.08 ± 0.04 relative to a value of 1.00 ascribed to the control F30 fraction. In contrast, insulin caused an approx. 30 % reduction in GLUT4 content in the F35 fraction, the immunoreactive GLUT4 signal falling from an arbitrary value of 1.00 (given to the control F35) to 0.73 ± 0.08 (values are means $\pm$ S.E.M. from three preparations). Assuming that the blotting efficiencies for F25, F30 and F35 were similar and taking account of the specific immunoreactivity of GLUT4 (densitometric units/mg of protein) and the total protein recovery of each of these fractions (F25, 30 ± 2 μg/g of muscle; F30, 53 ± 4 μg/g of muscle; F35, 150 ± 22 μg/g of muscle), then the relative pool sizes of GLUT4 in the three fractions, in the basal state, were (%): F25, 7 ± 1; F30, 44 ± 6; F35, 49 ± 7. These results indicate that, although the pool sizes of GLUT4 in F30 and F35 are very similar, only F35 contains the insulin-sensitive GLUT4 pool. These findings are consistent with those reported by Muñoz et al. [27,28], who have shown, using a subcellular fractionation procedure different from the one used in this study, that skeletal muscle contains GLUT4 populations that respond differentially to insulin. These observations support the existence of a large intracellular GLUT4 reservoir of which only a small fraction appears to be mobilized by insulin. Whether the remaining GLUT4 molecules within the F35 compartment are able to be regulated by factors other than insulin remains unknown. Interestingly, Coderre et al. [29] recently reported the existence of an exercise-sensitive intracellular GLUT4 pool which was independent of the one that responded to insulin. The fractionation protocol used by these authors was essentially the same as that described in this study but for the inclusion of additional sucrose-density layers. These authors found that the insulin-sensitive and the exercise-sensitive GLUT4 pools were recovered in membranes isolated from a 32 and 36 % sucrose fraction respectively, thus making it extremely unlikely that the GLUT4 compartment present in our endosome-enriched F30 fraction (recovered from the top of the 30 % sucrose cushion) resembles the exercise pool which they have characterized. It is possible, on the basis of the reported buoyant density of the membranes that house the exercise-sensitive GLUT4 pool [29], that our F35 fraction may harbour an exercise-responsive population of GLUT4 vesicles; however, previous studies have shown that exercise does not appear to affect the abundance of GLUT4 within F35 [30].

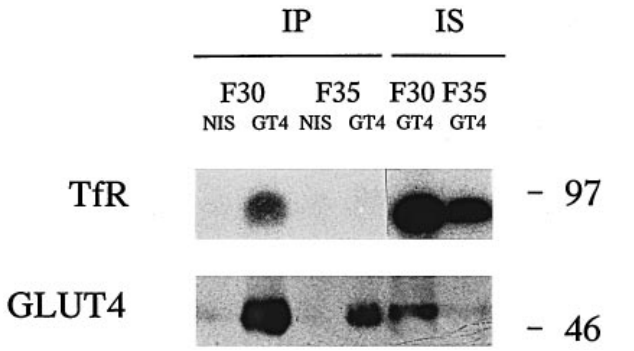

Figure 4 Immunoprecipitation of GLUT4-containing vesicles from F30 and F35 fractions

GLUT4-containing vesicles were immunoprecipitated from F30 and F35 fractions as described in the Materials and methods section using either anti-GLUT4 antibodies (GT4) or non-immune serum (NIS). The immune pellet (IP) and immune supernatant (IS) were screened by SDS/PAGE and Western blotting using antibodies to TfR and GLUT4.

Immunoisolation of the GLUT4-containing vesicles from the F30 and F35 fractions

The results shown above suggest that the GLUT4-containing vesicles present in the F30 and F35 fractions represent two pools of GLUT4, which exhibit differences in buoyant density and sensitivity to insulin. In order to assess whether we could discriminate between these two GLUT4 pools further, we immunoisolated GLUT4-containing vesicles from both these fractions. To this end, in initial experiments, a polyclonal anti-GLUT4 antibody was used and the overall efficiency with which the GLUT4 vesicles were precipitated from F30 and F35 were 49.4 ± 2.5 and 49.0 ± 1.1 % respectively (mean $\pm$ S.E.M., $n = 3$). In subsequent experiments the efficiency of immunoprecipitation of GLUT4 vesicles was significantly improved by using a monoclonal anti-GLUT4 antibody (see the Materials and methods section), which immunoadsorbed nearly 79 ± 2 and 77 ± 12 % of the total GLUT4 from F30 and F35 respectively. Interestingly, and regardless of which GLUT4 antibody was used, Western-blot analysis of GLUT4 vesicles isolated from F30 revealed the presence of TfR in the GLUT4 immunopellet. The degree of TfR/GLUT4 co-precipitation from three separate experiments was 27 ± 3 % (mean $\pm$ S.E.M.). This observation indicates that, although TfR co-localizes with GLUT4 in the endosomal compartment, a substantial proportion is present in endosomal vesicles that lack GLUT4. In contrast, GLUT4-containing vesicles isolated from the F35 fraction did not possess any immunoreactive TfR molecules, which we were able to detect in the F35 immune supernatant (Figure 4). However, since the entire F35 immune pellet and supernatant were loaded on to SDS-containing gels it is important to point out that the TfR signal observed in the immune supernatant is a consequence of having applied a substantial amount (~ 200 μg) of protein on to SDS-containing gels. It is not entirely appropriate therefore to compare data from Figure 4 with those shown in Figure 2 in which we observed very little TfR in the F35 fraction since in these experiments only 20 μg of protein was loaded on to the gels.

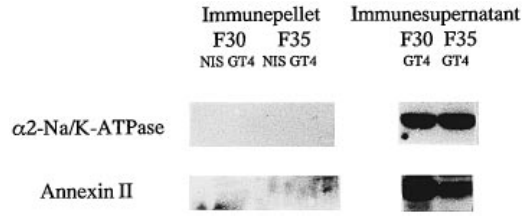

Figure 5 Lack of cell surface marker in the GLUT4-containing vesicles isolated from F30 and F35 fractions

GLUT4-containing vesicles were immunoprecipitated from F30 and F35 fractions as described in the Materials and methods section using either anti-GLUT4 antibodies (GT4) or a non-immune serum (NIS). The immune pellet and immune supernatant were screened by SDS/PAGE and Western blotting using antibodies to the α1-subunit of dihydropyridine receptor (a specific marker for the transverse tubules) and the $(Na^+ + K^+)$-ATPase α1 subunit and GLUT1, two well-established cell-surface markers in skeletal muscle.

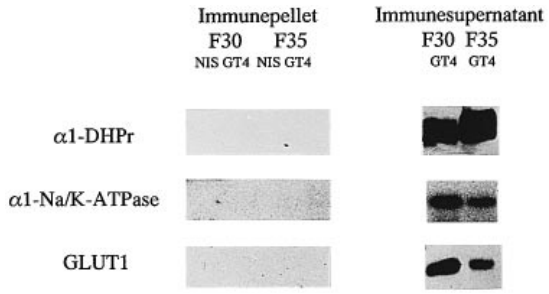

Figure 6 Demonstration that $(Na^+ + K^+)$-ATPase α2-subunit and annexin II, two proteins that are abundant in intracellular membranes of skeletal muscle, do not co-precipitate with GLUT4

GLUT4-containing vesicles were immunoprecipitated from F30 and F35 fractions as described in the Materials and methods section. The immune pellet and immune supernatant were screened by SDS/PAGE and Western blotting using antibodies to the $(Na^+ + K^+)$-ATPase α2 subunit and annexin II.

Although the co-localization of TfR and GLUT4 has previously been documented in 3T3-L1 adipocytes [8], to our knowledge the present data represent the first evidence that these proteins are also co-localized in an intracellular endosome-enriched muscle compartment. Three lines of evidence suggest that this result is not an artifact and that the TfR is a component of GLUT4 vesicles from the endosome-enriched fraction. First, the use of a non-immune serum does not immunoprecipitate GLUT4 or TfR. Secondly, although the TfR antigen was absent from the GLUT4-containing vesicles isolated from F35, when autoradiographic film was overexposed, the antigen could only be detected in the immune supernatant from the precipitation step (Figure 4). Finally, the presence of TfR in GLUT4 vesicles from F30 is unlikely to be the result of non-specific co-precipitation based on the finding that annexin II and the $(Na^+ + K^+)$-ATPase α2 subunit, which are also abundant in the F30 fraction, do not co-precipitate with GLUT4 (Figure 5). Furthermore three different cell-surface markers, GLUT1, the $(Na^+ + K^+)$-ATPase α1 subunit and the α1-subunit of the dihydropyridine receptor (the latter being a specific marker for the transverse tubules) were completely excluded from GLUT4 vesicles immunoprecipitated from either the F30 or F35 fractions (Figure 6). Our results showing the presence of specific endosomal markers (e.g. TfR) and the notable absence of others (e.g. annexin II) in GLUT4-containing vesicles isolated from the F30 fraction not only reflect, but underscore, the known structural and functional complexity of the endosomal compartment [25].

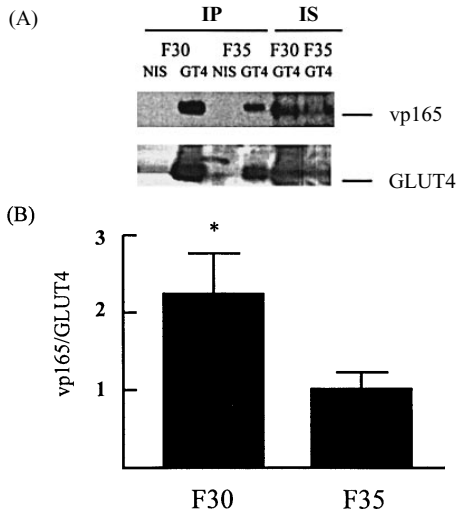

Figure 7 Different vp165/GLUT4 ratios in vesicles isolated from F30 and F35 fractions

(**A**) Representative Western blot showing vp165 abundance in GLUT4-containing vesicles isolated from F30 and F35 fractions. The immune pellets (IP) and immune supernatant (IS) obtained using either non-immune serum (NIS) or anti-GLUT4 antibody (GT4) were subjected to SDS/PAGE and Western blotting using antibodies to vp165 as described in the Materials and methods section. (**B**) Densitometric scanning data showing a higher vp165/GLUT4 ratio in the F30 GLUT4 vesicles than that found in the F35 GLUT4 vesicles. Autoradiograms were scanned and the densitometric signal for vp165 was divided by that obtained for GLUT4. A value of 1.00 was assigned to the compartment that displayed the lowest vp165/GLUT4 ratio. Data are means $\pm$ S.E.M. ($n = 4$), $^*P < 0.05$ by Student's t test.

The glycoprotein vp165 (also known as gp160) has been identified as a major protein component of the intracellular GLUT4 compartment in adipocytes [31,32]. vp165 has been recently cloned and characterized and shown to be a novel insulin-regulated membrane aminopeptidase [16,33]. Given the assertion that vp165 is closely associated with intracellular GLUT4, we investigated whether information about its distribution in the two intracellular GLUT4 pools could help to discriminate further between these compartments. Figure 7(A) shows that vp165 could be detected in GLUT4-containing vesicles immunoisolated from the F30 and F35 fractions. The degree of vp165 immunoadsorption from both fractions was $57 \pm 3\,\%$ and $64 \pm 10\,\%$ respectively (mean $\pm$ S.E.M.). However, quantitative analysis of data from four separate experiments showed that the relative abundance of vp165 (when expressed as a ratio of the signal intensity of vp165/GLUT4) was 2.2 ± 0.5-fold greater in vesicles isolated from the F30 fraction than the F35 fraction (Figure 7B).

To substantiate the observed biochemical co-localization of GLUT4 and vp165 we also performed immunogold double labelling on ultrathin sections of rat soleus muscle to assess whether these two proteins could be visualized on common membrane structures. In line with previous work [34–36], GLUT4 was detected in perinuclear regions (Figure 8A), along the junctions between the A and I bands where the transverse tubules run deep into the muscle fibre (Figure 8B) and in intermyofibrillar vesicles (Figure 8B). vp165 was also detected in perinuclear regions (Figure 8A) and on intermyofibrillar GLUT4-containing structures (Figure 8B) but the A–I junction was devoid of vp165 (Figure 8B). However, since the extent of vp165 labelling was low compared with that of GLUT4, this observation must be interpreted with caution. Moreover, owing to the low degree of vp165 labelling, it was not feasible to perform quantitative immunogold analyses of GLUT4/vp165 co-localization. Never-

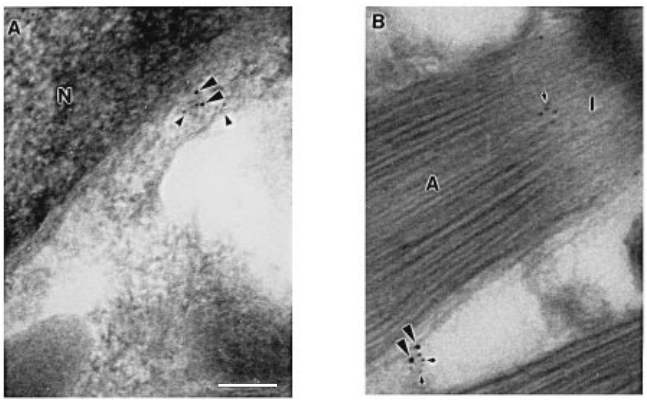

Figure 8 Electron-microscopic localization of GLUT4 and vp165 in rat soleus muscle

Electron micrographs of ultrathin cryosectioned soleus muscles labelled sequentially with anti-GLUT4 (5 nm gold particles; small arrowheads) and anti-vp165 (10 nm gold particles; large arrowheads) antibodies as described in the Materials and methods section. (**A**) Perinuclear co-localization of GLUT4 and vp165 near the nucleus (N); (**B**) intermyofibrillar vesicles containing GLUT4 and vp165. GLUT4 is also observed at the junction between myofibrillar A and I bands where transverse tubules are localized. One of these vesicles shows clear co-localization of GLUT4 and vp165. Bar represents 200 nm.

theless, the morphological results demonstrate for the first time that, in intact muscle, vp165 is present on intracellular membranes that also house the GLUT4 glucose transporter. The immunogold data are in good agreement with the immunoprecipitation analyses in suggesting that vp165 is present on two topologically different types of GLUT4-containing membrane structures: one near the nucleus close to the muscle surface and the other residing much deeper within the muscle fibre. Although it is tempting to speculate that the perinuclear vesicles and those located much deeper within the muscle may correspond to the biochemically characterized F30 and F35 fractions respectively, this suggestion remains tentative until more detailed information about the proteins present within these two vesicle populations and their morphological distribution in muscle has been obtained.

In summary, the present investigation has provided evidence for the isolation of two distinct intracellular GLUT4 pools in rat skeletal muscle. One of these GLUT4 pools lacks plasma membrane and endosomal protein markers but shows a reduction in its GLUT4 complement after insulin treatment. The membranous structures housing the second intracellular GLUT4 pool are less dense and may represent a pool that is constitutively recycling between the plasma membrane and early endosomes, based on the presence of the TfR in this compartment. Moreover, insulin does not induce any net change in GLUT4 content in this latter pool, although we are unable as yet to exclude the possibility that the hormone may induce changes in the turnover rate of GLUT4 molecules resident within this pool. Very little is currently know about the routing of the GLUT4 transporter to the plasma membrane. It remains unknown, for example, whether the increase in plasma-membrane GLUT4, occurring in response to insulin, is as a result of its recruitment directly from the specialized GLUT4-storage compartment or if it occurs *via* an endosomal/plasma-membrane recycling compartment. Further experimental analysis of the two intracellular GLUT4 pools described in this work may prove valuable in helping to define their functional roles with respect to subcellular GLUT4 trafficking in skeletal muscle.

Received 4 December 1996/17 March 1997; accepted 3 April 1997

We are grateful to Dr. V. Gerke (Münster, Germany) and Dr. K. Sweadner (Boston, MA, U.S.A.) for generously providing us with antibodies. J.C.A. was an MRC postdoctoral fellow. L. L. was supported by a postdoctoral fellowship from the Fonds de la Recherche en Santé du Québec (FRSQ). A.V. is supported by a predoctoral studentship from the Medical Research Council of Canada. This work was supported by research grants to A.K. from the Medical Research Council of Canada and to H.S.H. from the Medical Research Council (U.K.), The British Diabetic Association and The Wellcome Trust. H.S.H. is the recipient of a Wellcome Trust University Award.

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