Analyses of the co-localization of cellubrevin and the GLUT4 glucose transporter in rat and human insulin-responsive tissues

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Abstract **We have investigated the subcellular distribution and association of cellubrevin, a low molecular weight protein implicated in the process of membrane fusion, with intracellular membranes containing the insulin-sensitive GLUT4 glucose transporter from rat adipocytes, rat skeletal muscle and human skeletal muscle. SDS-PAGE and immunoblot analyses of subcellular fractions of adipocytes and skeletal muscle indicated a positive correlation between the distribution of GLUT4 and cellubrevin in intracellular membrane fractions tested from all tissues. The identity of the polypeptide reacting with antiserum against cellubrevin was further confirmed on the basis of its susceptibility to proteolysis by tetanus toxin. Immunoisolation of GLUT4-containing vesicles from a microsomal fraction enriched with GLUT4 and cellubrevin revealed that cellubrevin could be coprecipitated with GLUT4 vesicles from adipocytes. In contrast, intracellular GLUT4 vesicles isolated from both rat and human skeletal muscle were devoid of any detectable immunoreactivity towards cellubrevin. The observation that cellubrevin does not colocalise with intracellular GLUT4 in skeletal muscle from two different species, rat and human, would strongly suggest that it is unlikely to participate in the insulin-induced delivery of GLUT4 to the cell surface in skeletal muscle.**

Key words: SNARE; Membrane; Transport; Muscle; Adipocyte

1. Introduction

In skeletal muscle and fat, insulin causes rapid stimulation in glucose transport which occurs largely as a result of an increase in the number of functional GLUT4 glucose transporters in the plasma membrane in response to hormone binding. In the basal or unstimulated state, the bulk of the cellular GLUT4 resides in specialized intracellular storage vesicles which can be mobilized upon exposure of adipose tissue and skeletal muscle to insulin [1–3]. Whilst there is firm acceptance of the GLUT4 translocation hypothesis, information concerning the cellular machinery involved in the insulin-induced trafficking and fusion of GLUT4-containing vesicles with the plasma membrane remains extremely limited at present. However, it is not unreasonable to assume that a common group of proteins may be involved in the basic steps of intracellular membrane movement, docking and fusion with a target membrane in most cell types. The SNARE hypothesis provides an appealing framework for understanding the mechanism of vesicle targeting and fusion [4,5]. In this model, vesicle docking and fusion relies upon the pairwise matching of specific small membrane proteins present on the cytoplasmic surface of the transport vesicle (denoted a v-SNARE) and on the target membrane (denoted a t-SNARE). A number of proteins have now been identified as members of the v-SNARE class [6–8]. Two proteins belonging to this family, VAMP2 and cellubrevin, have recently been shown to be expressed in rodent muscle [9,10] and adipocytes [11,12] and their abundance increases significantly upon cellular differentiation [9,12], suggesting that they may participate in regulating membrane trafficking events associated with the differentiated phenotype. Cellubrevin is considered to reside largely in recycling endosomes rather than specialized secretory vesicles [7]. Given that the GLUT4 transporter has also been shown to recycle to and from the cell surface, a process that is acutely regulated by insulin [13,14], and the finding that cellubrevin is a resident protein in GLUT4 vesicles isolated from murine 3T3-L1 adipocytes [12], the possibility exists that cellubrevin may be involved in the delivery and fusion of GLUT4 vesicles with the plasma membrane in insulin-sensitive tissues.

No information is currently available concerning the expression of cellubrevin in human skeletal muscle. Furthermore, although evidence exists showing that cellubrevin is expressed in rat skeletal muscle, it remains presently unknown whether it is associated with intracellular vesicles containing GLUT4 and consequently whether it participates in the translocation of GLUT4 in this tissue. Addressing this question is of some importance since, unlike adipose tissue, skeletal muscle makes a quantitatively larger contribution towards insulin-stimulated glucose disposal [15] and also represents the major site of insulin resistance in non-insulin-dependent diabetes [16]. Knowledge of the mechanisms involved in the trafficking of GLUT4 will thus not only be of general biochemical interest but may also prove invaluable in understanding the pathogenesis of impaired glucose utilization. In this study, we present novel evidence showing that, despite the expression of cellubrevin in rat and human skeletal muscle, it appears not to be a component of muscle GLUT4 vesicles isolated from insulin-responsive fractions. This observation strongly contrasts with our finding that cellubrevin colocalises with GLUT4 in rat adipocytes. The implications of these observations are discussed.

2. Materials and methods

2.1. Tissue procurement

Human soleus muscle (20–30 g) was obtained from patients at the Orthopaedic Department of King Cross Hospital, Dundee undergoing elective limb amputation surgery due to peripheral vascular complications. Upon surgical excision, ~0.5 g of muscle was reserved for histochemical analyses and the remainder, intended for subcellular fractionation studies, was immediately frozen in liquid nitrogen and stored at −80°C until required for study. Only skeletal muscle that showed no signs of necrosis and which was judged to be well perfused

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and oxygenated, and which subsequently stained histochemically normal for myosin-ATPase, was used in the present study. For rat tissues, male Sprague Dawley rats (200–250 g) were killed by cervical dislocation and epididymal fat pads and hindlimb skeletal muscle rapidly excised. Isolated fat pads were used immediately for study, whereas, hindlimb muscle was frozen in liquid nitrogen and stored at −80°C until required.

2.2. Subcellular fractionation of isolated tissue

Rat adipose tissue was subjected to 0.1% collagenase digestion at 37°C for 45 min. Digested tissue was subsequently filtered through nylon mesh (180 μm) for isolation of adipocytes which were subsequently fractionated according to the method described by Simpson et al. [17]. The procedure allows the isolation of fractions enriched with plasma membranes (PM), low density microsomes (LDM) and high density microsomes (HDM) [17].

Rat and human skeletal muscle was homogenized and subjected to differential centrifugation for isolation of crude (total) muscle membranes which were subfractionated on a discontinuous sucrose density gradient (25, 30 and 35%) as described previously [18–20]. This procedure results in the separation of three distinct membrane bands; one on top of the 25% sucrose cushion (F25) representing membranes enriched with PM markers [19–21], a second band separates on top of the 30% sucrose layer (F30) containing intracellular membranes largely of endosomal origin [22] and a third band separates out on top of the 35% sucrose layer (F35) and is composed of membranes endowed with the insulin sensitive pool of GLUT4 [3,23]. The protein content of individual adipocyte and muscle fractions was determined using the method of Bradford [24].

2.3. Western-blot analyses

Adipocyte and muscle membrane fractions (20 μg protein) were resolved by SDS-PAGE as described by Laemmli [25]. Samples were transferred onto nitrocellulose filters, blocked and incubated overnight at 4°C with antiserum specific for GLUT4 (1:500, East Acres Biologicals, Southbridge, MA, USA). Western blots of cellubrevin (using either a 1:500 dilution of MC16-antibody [26] kindly provided by Dr. Pietro De Camilli, Yale University School of Medicine, New Haven or a 1:1000 dilution of D204 antibody [7] gifted by Dr. Harvey McMahon, MRC Unit, Cambridge, UK) were carried out on membrane samples which had previously been resolved by SDS-PAGE using Schagger-type gels [27] which allows better separation of proteins in the low molecular weight range. Primary antibody detection was carried out using either 0.1 μCi/ml [^{125}I]Protein A or a horseradish-peroxidase-conjugated secondary antibody and enhanced chemiluminescence.

2.4. Sensitivity of cellubrevin to tetanus toxin

The susceptibility of cellubrevin expressed in membrane fractions isolated from rat muscle and fat to tetanus toxin was investigated by incubating intracellular muscle membranes isolated from the F30 and F35 and from the LDM adipocyte fraction with 20 μg/ml whole tetanus toxin (kindly provided by Dr. Colin Watts, Department of Biochemistry, University of Dundee), which had been preactivated by incubation with 10 mM dithiothreitol, 50 mM NaCl, 0.25 mM $ZnCl_2$, 10 mM HEPES, pH 7.2. Membranes and toxin were incubated at 37°C for 1 h and the reaction terminated by the addition of Laemmli buffer and boiling of samples for 3 min. In some experiments 0.5%Triton X-100 was included during the toxin incubation period to assess if the toxin's ability to cleave cellubrevin was enhanced. Samples were then resolved by SDS-PAGE on Schagger-type gels and Western blotting using antiserum to cellubrevin performed as described above.

2.5. Immunoisolation of intracellular GLUT4-containing vesicles

The procedure for immunoisolating GLUT4-containing vesicles from rat skeletal muscle and fat microsomes has been previously described in detail [23,28]. Briefly, 200–300 μg of membrane protein from the F30 and F35 muscle fraction or from LDM (adipocytes) were treated with 5 μl of non-relevant serum or 5 μl of anti-GLUT4 serum (East Acres, Southbridge, MA). The GLUT4-containing vesicles were complexed by the addition of Protein A-Sepharose beads and pelleted by centrifugation. The resulting supernatant was centrifuged at 190 000 × *g*, the pellet from this step being resuspended in Laemmli buffer and termed the immunesupernatant. The immunopellet was washed twice with phosphate-buffered saline (PBS, pH 7.4) prior to a single wash with 1 M NaCl, 1 mM EDTA and 50 mM HEPES pH 7.4 followed by two final washes in PBS before resuspension in Laemmli buffer. For the immunoisolation of GLUT4-containing vesicles from human skeletal muscle a different procedure was followed. Purified anti-GLUT4 antibody 1F8 (Genzyme) as well as non-specific mouse IgG (Sigma) were coupled to Protein G-Sepharose beads (GammaBind Plus Sepharose, Pharmacia) at a concentration of 1 mg/ml beads. The antibodies were then cross-linked to the beads using dimethyl pimelimidate as described elsewhere [29]. Between 200 and 300 μg of membrane protein from the F30 and F35 human muscle fractions, in a final volume of 200 μl, were treated with 20 μg of 1F8 or non-specific mouse IgG previously cross-linked to the beads as described above. After 1 h incubation at room temperature the samples were applied to a discontinuous sucrose gradient (10 and 40% sucrose w/w) and centrifuged at 190 000 × *g* for 30 min. The membranes recovered from the top of the 40% sucrose band were diluted with PBS and pelleted at 190 000 × *g*, the pellet from this step was resuspended in Laemmli buffer and termed the immunesupernatant. The beads and vesicles bound to them were collected from the bottom of the sucrose gradient tube and washed twice with PBS prior to a single wash with 1 M NaCl, 1 mM EDTA and 50 mM HEPES pH 7.4 followed by two final washes in PBS. The adsorbed material was eluted with Laemmli buffer.

3. Results

3.1. Subcellular distribution of cellubrevin in rat muscle and adipocytes

The distribution of cellubrevin in membrane fractions isolated by subcellular fractionation from both skeletal muscle and primary rat adipocytes is shown in Fig. 1. Quantitative analyses of cellubrevin immunoreactivity in the various fractions from three individual experiments revealed that its relative distribution in the F25, F30 and F35 muscle fractions was 21 ± 4, 52 ± 2 and 27 ± 9%, respectively. PM, LDM and HDM fractions isolated from adipocytes showed that the relative distribution of cellubrevin was 29 ± 7, 49 ± 1 and 22 ± 4%, respectively. The observed subcellular distribution of cellubrevin in both tissues was identical irrespective of whether we used the MC16 or D204 anti-cellubrevin antibodies. The observed enrichment of cellubrevin in the LDM fraction of adipocytes is of interest, since this fraction also houses the insulin-responsive pool of GLUT4 glucose transporters [30]. In skeletal muscle GLUT4 is abundant in membranes from both the F30 and F35 but only the latter fraction contains the translocatable pool of GLUT4 [3,23]. However, cellubrevin reactivity

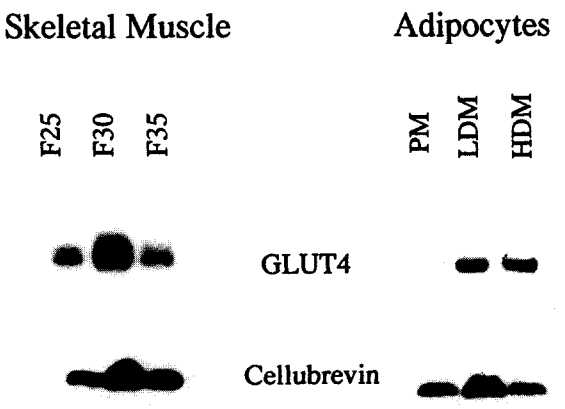

Fig. 1. Representative Western blots showing the distribution of GLUT4 and cellubrevin in rat skeletal muscle and adipocyte subcellular fractions. Proteins (20 μg) from each fraction were subjected to SDS-PAGE and immunoblotting as described in the text.

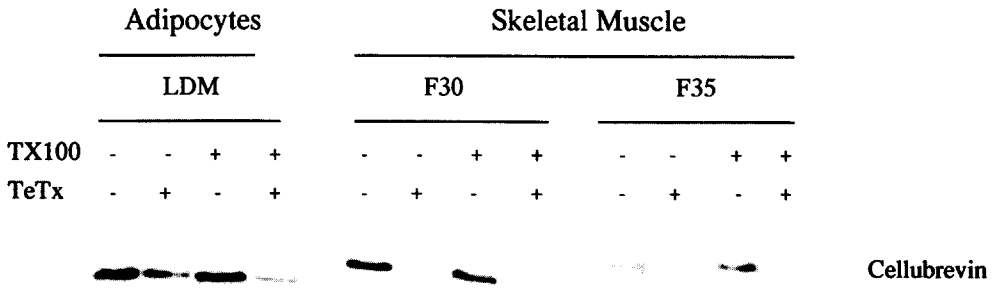

Fig. 2. Effects of tetanus toxin on cellubrevin in rat skeletal muscle and adipocytes. Isolated membrane fractions from both skeletal muscle and adipocytes, were incubated with or without tetanus toxin (20 μg/ml) as described in Section 2. The incubation of membranes and toxin was performed in the absence or presence of 0.5% Triton X-100. After a 1 h in vitro incubation, samples were boiled for 3 min in Laemmli sample buffer and subjected to SDS-PAGE and immunoblotting using cellubrevin antibodies.

was found to be most enriched in muscle membranes from the F30 which are largely of endosomal origin [22].

3.2. Susceptibility of cellubrevin to proteolysis by tetanus toxin

In vitro studies have shown that cellubrevin is a good substrate for tetanus toxin [7]. To confirm further that the antigen reacting with the anti-cellubrevin antibodies used in this study was indeed cellubrevin, we assessed the integrity of the immunoreactive band following in vitro treatment of LDM adipocyte membranes and intracellular muscle membranes (from the F30 and F35) with tetanus toxin. Fig. 2 shows that exposure of the different muscle and fat membrane fractions to toxin led to a significant reduction in cellubrevin immunoreactivity. We also investigated the effects of 0.5% Triton X-100 during the incubation period of the membranes with toxin since it has been suggested that mild detergent treatment may facilitate proteolysis of cellubrevin by increasing the accessibility of the toxin to the cleavage site [31]. However, we were unable to observe any significant differences in cellubrevin degradation as a result of detergent treatment. The effect of the toxin was selective for cellubrevin as the immunoreactivity of GLUT4 within the same membrane fractions was unaffected by the toxin (data not shown). The specificity of the toxin for cellubrevin was further confirmed by the lack of any detectable differences in the overall protein composition of the various fractions as judged by analyses of gels stained with Coomassie blue (data not shown).

3.3. Analyses of the localization of cellubrevin in GLUT4-containing vesicles isolated from rat muscle and adipocytes

The observed enrichment of cellubrevin in intracellular fractions of rat skeletal muscle and adipocytes, that were also enriched with GLUT4 (Fig. 1), raises the possibility that the two proteins may be resident on common intra-vesicular structures. To test this proposition we immunoprecipitated GLUT4-containing vesicles from the insulin-sensitive fractions (LDM from adipocytes and the membranes from the F35 rat muscle fraction), as well as the from the F30 rat muscle fraction which is enriched with endosomal membranes [22]. The immune pellets (containing the GLUT4 vesicles) and the corresponding immunesupernatants from these fractions were screened using antibodies specific for cellubrevin. In four independent experiments for muscle and three for adipocytes, the efficiencies with which GLUT4 vesicles were precipitated, when using a polyclonal anti-GLUT4 antibody, were

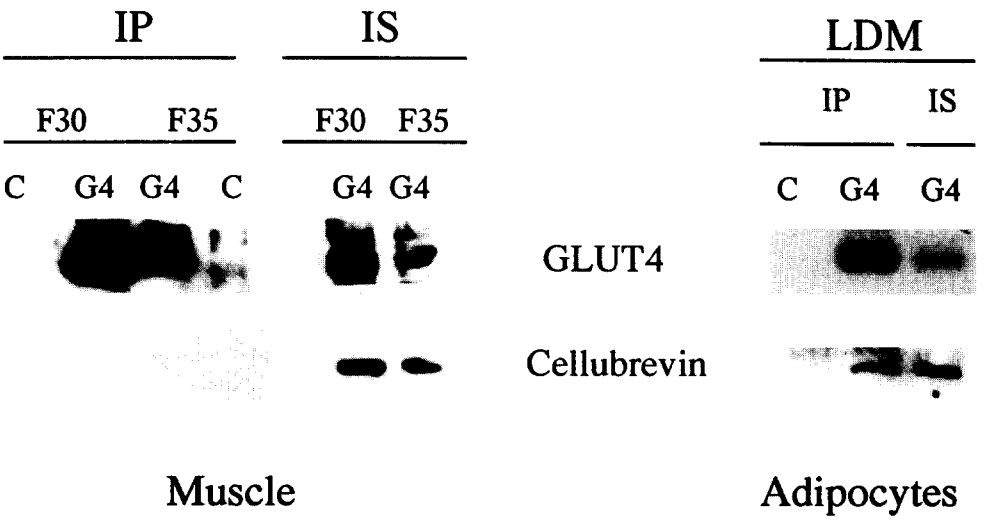

Fig. 3. Analyses of the localization of cellubrevin in GLUT4-containing vesicles. Intracellular membranes fractions (LDM from adipocytes and membranes from the F30 and F35 rat muscle fractions) were incubated with a control non-immune (C) IgG or with anti-GLUT4 (G4) antibody, and immunoprecipitated as described in Section 2. The immunopellets (IP) and the corresponding immunesupernantants (IS) from these fractions were screened using antibodies specific for GLUT4 and cellubrevin.

61 ± 9, 49 ± 2 and 48 ± 2% from the LDM (adipocytes), F30 and F35 muscle fractions, respectively. However, precipitation of GLUT4 vesicles from the rat muscle fractions was significantly improved using a monoclonal anti-GLUT4 (1F8) antibody. 1F8 immunoadsorbed nearly 79 and 77% of the total GLUT4 from the F30 and F35, respectively. Regardless of which GLUT4 antibody was used for the immunoisolation of muscle GLUT4 vesicles we were unable to detect any immunoreactivity towards cellubrevin in the GLUT4 immune pellets (Fig. 3). However, immunoprecipitation of GLUT4 vesicles from the LDM fraction of rat adipocytes resulted in the coprecipitation of cellubrevin. Use of a non-immune rabbit serum as control did not result in precipitation of GLUT4 or cellubrevin (Fig. 3).

3.4. *Expression and subcellular distribution of cellubrevin in human skeletal muscle*

The distribution of cellubrevin in human muscle membranes isolated by our fractionation procedure is shown in Fig. 4. Quantitative densitometry of immunoblot data from three separate human muscle preparations revealed that the relative distribution of cellubrevin in the F25, F30 and F35 was 48 ± 11, 33 ± 5 and 19 ± 10% (mean ± S.E.M.), respectively. We have previously reported that the F25 human fraction is enriched with PM-markers (ie. the GLUT5 hexose transporter [19] and the α_1-subunit of the Na,K-ATPase [20]) but that both these markers are not detectable in the F30 or F35 signifying that membranes from these two fractions are likely to be largely of intracellular origin [19,20]. Of interest was the finding that, as in rat skeletal muscle (Fig. 1), the human F30 muscle fraction housed significant amounts of both cellubrevin and GLUT4 (Fig. 4). We therefore investigated whether these proteins were associated by immunoprecipitating GLUT4 vesicles from the F30 and from the F35 (which also contains significant amounts of GLUT4) and screened the isolated GLUT4 vesicles with anti-cellubrevin antibodies. From three separate immunoprecipitation experiments the efficiency with which GLUT4 vesicles were isolated from the F30 and F35 were 86 ± 2 and 89 ± 7% (mean ± S.E.M.), respectively. Analyses of immunoisolated GLUT4 vesicles from both fractions revealed that they did not contain any detectable reactivity against cellubrevin which was fully recovered in the immunesupernatant (Fig. 5). The observed segregation of GLUT4 and cellubrevin in human skeletal muscle is in full agreement with the data obtained from using rat skeletal muscle (Fig. 3).

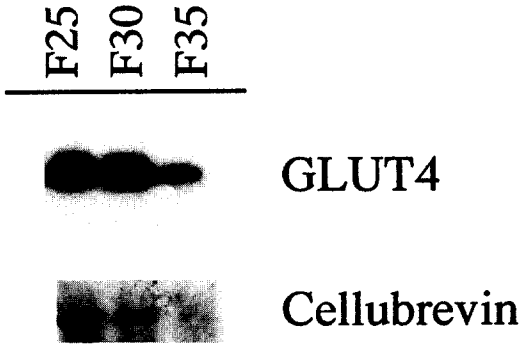

Fig. 4. Representative Western blots showing the distribution of GLUT4 and cellubrevin in human skeletal muscle fractions. Proteins (20 μg) from each membrane fraction were subjected to SDS-PAGE and immunoblotting as described in the text.

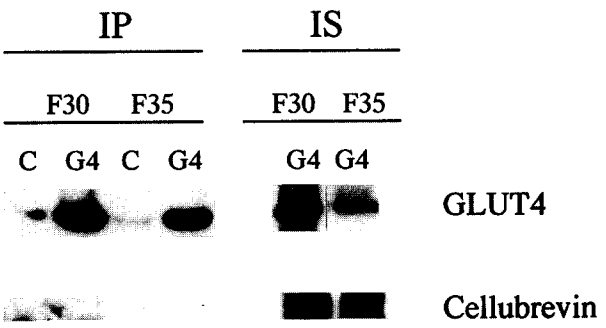

Fig. 5. Analyses of the localization of cellubrevin in GLUT4-containing vesicles immunoprecipitated from human skeletal muscle. Intracellular membrane fractions (F30 and F35) were incubated with a control non-immune (C) IgG or with 1F8 anti-GLUT4 (G4) antibody, and immunoprecipitated as described in Section 2. The immunopellets (IP) and the corresponding immunesupernantants (IS) from these fractions were screened using antibodies specific for GLUT4 and cellubrevin. It should be noted that the strong cellubrevin reactivity observed in the IS from the F35 is as a consequence of using 10–15 fold more protein than that used to assess cellubrevin distribution in the different muscle fractions shown in Fig. 4.

4. Discussion

It is now well established that the expression of the GLUT4 glucose transporter in skeletal muscle and fat forms an integral component of the mechanism by which insulin is able to acutely stimulate the uptake of glucose in these tissues (for reviews see [32–34]). However, data from a number of cell transfection studies clearly indicate that the expression or presence of GLUT4 transporters and insulin receptors alone do not fulfil the basic requirements for insulin-dependent glucose transport (reviewed in [34]). Transfection of GLUT4 into cells which normally do not express this transporter isoform has invariably led to the observation that, whilst the transporter may be efficiently expressed and retained in a cytoplasmic compartment, insulin fails to induce its translocation to the cell surface in a manner observed in muscle and fat cells. It has been suggested that the inability of insulin to stimulate GLUT4 translocation in transfected cell lines may reflect the need to have present additional gene products whose expression may be central to the vesicle trafficking and fusion process.

In an attempt to understand the cellular components involved in the insulin-induced recruitment of GLUT4 there has been growing interest towards the analyses and identification of proteins associated with intracellular vesicles enriched with GLUT4 in fat and skeletal muscle. In particular, recent attention has focussed upon a low molecular weight protein called cellubrevin which belongs to the v-SNARE family. Studies in 3T3-L1 adipocytes have revealed that this murine cell line expresses cellubrevin and more importantly that it appears to be a constituent protein of GLUT4 vesicles isolated from the insulin-responsive microsomal compartment. The presence of a v-SNARE in these isolated vesicles provides a strong basis for suggesting that it may participate in events related to the fusion of GLUT4 membrane vesicles [12]. However, owing to technical difficulties normally associated with

fractionation and isolation of GLUT4 vesicles from skeletal muscle, information on whether cellubrevin may participate in the trafficking of muscle GLUT4 has been less forthcoming. Using subcellular fractionation and immunoprecipitation methods that are well established in our hands, the present study has attempted to address this issue through a comparative analyses of GLUT4 vesicles isolated from rat adipose tissue and rat and human skeletal muscle. Our observations showing the presence of cellubrevin in GLUT4-containing vesicles isolated from the light microsomal fraction of primary rat adipocytes are consistent with those made in 3T3-L1 adipocytes [12] and may signify that the colocalization of cellubrevin and GLUT4 is a hallmark of the fat cell phenotype which is not influenced by the cells genotypic origin.

Analyses of GLUT4 vesicles from both adipocytes and muscle have led to the identification and characterization of a number of proteins including vp165 or gp160 [35,36], SCAMPs [37,38] and Rab4 [23,39]. Based on the observation that all of these proteins are common to GLUT4 vesicles isolated from muscle and fat [23,39,40] and recent work showing that the insulin-responsive GLUT4 vesicles from both tissues have comparable sedimentation velocity rates, buoyant densities and protein staining patterns it has been suggested that they are likely to be functionally similar [40]. However, our finding that cellubrevin does not colocalise with intracellular GLUT4 in skeletal muscle represents, to our knowledge, the first reported discrepancy in the polypeptide composition between GLUT4-containing vesicles isolated from rat muscle and adipose tissue. It is likely that these differences have been identified partly as a result of isolating distinct subcellular populations of GLUT4 vesicles from skeletal muscle. The notion that multiple intracellular GLUT4 pools may exist and that such pools are likely to be compositionally different is gaining more widespread acceptance [22,41,42]. Indeed, our observation that cellubrevin does not colocalise with muscle GLUT4 in the insulin-responsive pool (F35) is in full agreement with recent findings made by Zorzano's group who have also found, using an entirely different muscle fractionation protocol, that cellubrevin is not localised with GLUT4 in the intracellular pool that responds acutely to insulin in their hands. Their protocol, however, does allow them to isolate another sub-population of GLUT4 vesicles that are insensitive to insulin [43], but which appear to harbor detectable amounts of cellubrevin (personal communication). The precise significance of this latter finding remains unknown at present, but given our finding (and the unpublished observations of Zorzano's group) that cellubrevin is not a component of the insulin-sensitive GLUT4 pool in skeletal muscle would strongly indicate that it is unlikely to play any direct role in the insulin-induced delivery of GLUT4 to the cell surface.

The insulin-induced translocation of GLUT4 in human skeletal muscle has previously been documented [44,45] but no information currently exists concerning the nature or composition of intracellular GLUT4 vesicles from human skeletal muscle. The lack of any progress on this point has largely been due to the inability to obtain sufficient quantities of human skeletal muscle for subcellular fractionation and immunoprecipitation analyses. The present work therefore represents a major step in terms of overcoming these difficulties and reporting, for the first time, the successful isolation of GLUT4 vesicles from human skeletal muscle which may now be characterised in detail. Our findings show that whilst the human F30 fraction is enriched with both cellubrevin and GLUT4, isolation of GLUT4 vesicles from this fraction does not result in the coprecipitation of cellubrevin. Similarly, GLUT4 vesicles isolated from the human F35 (the cognate F35 rat muscle fraction contains the insulin responsive GLUT4 pool) are also devoid of cellubrevin. These findings are consistent with those made in rat skeletal muscle and strengthen the suggestion that cellubrevin is not likely to be involved in the targetting of GLUT4 to the cell surface in either rat or human skeletal muscle.

In stark contrast, cellubrevin is a component of intracellular GLUT4 vesicles isolated from rat adipocytes and this raises the interesting possibility that if it is involved in the insulin-regulated movement and fusion of GLUT4 vesicles in adipose tissue, then the mechanism may be one that is functionally different to that operating in skeletal muscle. In an attempt to gain some insight into this issue we are currently investigating the effects of ablating cellubrevin expression in fat cells with a view to evaluating its importance in the insulin-induced stimulation of glucose transport and GLUT4 translocation.

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