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The glucose transporter GLUT4 and the aminopeptidase vp165 colocalise in tubulo-vesicular elements in adipocytes and cardiomyocytes

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SUMMARY

The aminopeptidase vp165 is one of the major polypeptides enriched in GLUT4-containing vesicles immuno-isolated from adipocytes. In the present study we have confirmed and quantified the high degree of colocalisation between GLUT4 and vp165 using double label immuno-electron microscopy on vesicles isolated from adipocytes and heart. The percentage of vp165-containing vesicles that also contained GLUT4 was 91%, 76%, and 86% in rat adipocytes, 3T3-L1 adipocytes, and rat heart, respectively. Internalisation of a transferrin/HRP (Tf/HRP) conjugate by 3T3-L1 adipocytes, followed by diaminobenzidine treatment in intact cells, resulted in ablation of only 41% and 45% of GLUT4 and vp165, respectively, whereas endosomal markers are almost quantitatively ablated. Using immuno-electron microscopy on cryosections it was determined that in atrial cardiomyocytes GLUT4 and vp165 colocalised in a population of tubulo-vesicular (T-V) elements that were often found close to the plasma membrane. Double label immunocytochemistry indicated a high degree of overlap in these T-V elements between GLUT4 and vp165. However, in atrial cardiomyocytes a large proportion of GLUT4 was also present in secretory granules containing atrial natriuretic factor (ANF). In contrast, very little vp165 was detected in ANF granules. These data indicate that GLUT4 and vp165 are colocalised in an intracellular, post-endocytic, tubulo-vesicular compartment in adipocytes and cardiomyocytes suggesting that both proteins are sorted in a similar manner in these cells. However, GLUT4 but not vp165 is additionally localised in the regulated secretory pathway in atrial cardiomyocytes.

Key words: Insulin action, Protein targeting

INTRODUCTION

Insulin stimulates glucose transport in adipose tissue and muscle through the translocation of a glucose transporter isoform, GLUT4, from an intracellular site to the cell surface (Bell et al., 1993; James et al., 1994). Fundamental to this response is the efficient intracellular sequestration of GLUT4. In order to understand the mechanism by which GLUT4 is excluded from the cell surface in the absence of insulin, and how insulin stimulates the translocation of GLUT4, it is essential to define the nature of the intracellular compartment(s) in which it resides. GLUT4 has been localised in adipocytes and muscle by electron microscopy (Slot et al., 1991a,b; Rodnick et al., 1992). Although the transporter is found in endosomes and in the *trans*-Golgi reticulum (TGR) the majority is located in a tubulo-vesicular (T-V) compartment. In an effort to characterise this compartment a number of laboratories have immuno-isolated GLUT4-containing vesicles and studied their constituent proteins. The majority of the proteins found to date are of endosomal origin. These include the mannose 6-phosphate receptor (Tanner and Lienhard, 1989), the transferrin receptor (TfR) (Tanner and Lienhard, 1989; Hanpeter and James, 1995), transferrin (Hanpeter and James, 1995) and cellubrevin (Volchuk et al., 1995; Martin et al., 1996). However, one of the major constituents, at least as determined by protein staining, is a 165 kDa protein which has been characterised as an aminopeptidase (Kandror and Pilch, 1994; Mastick et al., 1994; Kandror et al., 1994; Keller et al., 1995). This protein is unique compared to most other co-inhabitants of GLUT4-containing vesicles because it has a very similar distribution and insulin regulation to GLUT4 (Ross et al., 1996).

Results obtained previously using an endosomal ablation technique, in conjunction with immuno-electron microscopy (immuno-EM) on vesicles isolated from adipocytes, has enabled us to resolve two separate GLUT4 compartments in adipocytes (Livingstone et al., 1996; Martin et al., 1996). One compartment comprised approximately 40% of intracellular GLUT4 and contained several endosomal markers including the mannose 6-phosphate receptor, the TfR and cellubrevin. The second compartment, comprising the remaining GLUT4, was devoid of endosomal markers but contained VAMP2, a synaptobrevin isoform also found in small synaptic vesicles (Schiavo et al., 1992; Südhof et al., 1993). In the present study

we have used a variety of morphological techniques, as well as the endosomal ablation technique, to compare the distribution of vp165 and GLUT4 in adipocytes and cardiomyocytes. The results suggest that these two proteins have very similar, but not identical, targeting properties in adipocytes and cardiomyocytes.

MATERIALS AND METHODS

Materials

HRP-conjugated goat anti-rabbit IgG and enhanced chemi-luminescence (ECL) detection kits were from Amersham (Aylesbury, UK). All tissue culture reagents were from Gibco BRL, with the exception of foetal calf serum which was from CSL (Commonwealth Serum Laboratories, Parkville, Australia). Poly(vinylidene difluoride) blotting membranes were from Millipore (Massachusetts, USA). BCA protein assay kits were from Pierce (Rockford, Illinois, USA). Cy3-conjugated goat anti-rabbit IgG was obtained from Jackson ImmunoResearch Labs (West Grove, PA, USA). Protein A-gold was prepared as previously described (Roth et al., 1978; Slot and Geuze, 1985). All other chemicals were of high quality commercial grades.

Antibodies

Antisera raised against a synthetic peptide corresponding to the C-terminal 12 amino acids of GLUT4 (James et al., 1989) has been described previously. Antibodies raised against a fusion protein consisting of the cytoplasmic N-terminal domain (amino acids 2-109) of vp165 and glutathione S-transferase, and affinity-purified using the fusion protein coupled to a glutathione-Sepharose column, have also been described previously (Keller et al., 1995). Antiserum raised against the N terminus of pro-ANF was a gift from Dr Gaétan Thibault (ICRM, Montreal, Canada).

Preparation of cells and tissues

3T3-L1 fibroblasts (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% new-born calf serum, 2 mM L-glutamine, 100 units/l penicillin and 100 mg/l streptomycin at 37°C in an atmosphere of 5% CO_2. Cells were passaged at sub-confluency. Confluent cells were differentiated into adipocytes as described (Frost and Lane, 1985). Cells were used at day 8 post-differentiation. To establish basal conditions prior to use, 3T3-L1 cells were incubated for 2 hours in serum-free DMEM.

Primary adipocytes were prepared from the epididymal fat pads of male Wistar rats by the collagenase digestion technique (Simpson et al., 1983). Following isolation, the adipocytes were incubated in modified KRP (12.5 mM Hepes, 120 mM NaCl, 6 mM KCl, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM sodium phosphate, 2 mM sodium pyruvate, 2% BSA, pH 7.4) for 30 minutes to establish basal conditions.

Total membrane fractions were prepared from rat tissues, primary rat adipocytes, 3T3-L1 adipocytes and 3T3-L1 fibroblasts as described previously (Martin et al., 1996). Subcellular fractionation of 3T3-L1 adipocytes was performed as described by Piper et al. (1991).

Immunolabelling of cryosections and isolated vesicles

Male Wistar rats were fasted overnight with free access to water. Perfusion fixation, using 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 5 minutes, was performed as previously described (Slot et al., 1991a) on animals anaesthetised with pentobarbitone. Hearts were then collected and fixation was prolonged by immersing the tissue samples in fixative for a further 1-2 hours at room temperature. Fixed hearts were stored in 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C.

Ultracryomicrotomy was performed by a slight modification of the Tokuyasu technique (Tokuyasu, 1980). The atria were dissected from the hearts. Atrial blocks (~1 mm^3) were rinsed briefly in PBS, then incubated in 2.3 M sucrose for 2 hours at room temperature. All blocks were then mounted on specimen holders and frozen in liquid nitrogen. For fluorescence microscopy semi-thin sections (~350 nm) were cut at −90°C, and for electron microscopy ultra-thin sections (~55 nm) were cut at −120°C, using a Leica Ultracut S/FCS cryomicrotome (Leica Instruments Pty Ltd, North Ryde, Australia) equipped with a Diatome anti-static device (Diatome, Biel, Switzerland), and a diamond knife designed for ultracryotomy (Drukker, Cuyck, The Netherlands). Sections were picked up and thawed on the surface of a drop of 1.15 M sucrose/1% methylcellulose (Liou et al., 1996). Semi-thin sections were then transferred to poly-L lysine coated glass slides, and ultra-thin sections transferred to Formvar carbon-coated copper grids.

Semi-thin sections were immunolabelled for fluorescence microscopy using Cy3-conjugated goat anti-rabbit IgG as a fluorescent marker. Prior to labelling of ultra-thin tissue sections the sucrose/methylcellulose was diffused from the surface of the grids by incubation on a plate of solid 2% gelatin in 0.1 M phosphate buffer, pH 7.4, containing 0.05% sodium azide, until the gelatin had liquefied. Immunolabelling was then performed as described previously (Martin et al., 1996). Grids were dried and viewed using a Jeol 1010 transmission electron microscope.

Heart from rats fed ad libitum was finely chopped in 2× 20 mM Hepes, 250 mM sucrose, 1 mM EDTA, pH 7.4 (HES buffer), containing 10 μg/ml leupeptin, 10 μg/ml aprotinin, 250 μM PMSF, and homogenised thoroughly using a Polytron homogeniser. Vesicles were prepared from the heart homogenate, and from 3T3-L1 adipocyte and rat adipocyte homogenates as described previously (Martin et al., 1996). Fixed membrane vesicles were stored at 4°C. Immunolabelling of vesicles was performed as described previously (Martin et al., 1996).

Endosome ablation

The Tf/HRP was prepared using the carbodiimide method of Kishida et al. (1975). The use of the Tf/HRP conjugate for ablation of endosomes in 3T3-L1 adipocytes has been described in detail in previous studies (Livingstone et al., 1996; Martin et al., 1996). Before use, the conjugate was iron-loaded as described by West et al. (1994). Basal 3T3-L1 adipocytes were incubated with the Tf/HRP for the times and at the temperatures indicated in the Results. Cells were then transferred to ice, and the diaminobenzidine (DAB) cytochemistry reactions performed in the absence or presence of H_2O_2. Cells were washed once with PBS containing 5 mg/ml BSA and three times with ice-cold HES buffer, prior to subcellular fractionation.

SDS-PAGE and western blotting

SDS-PAGE was performed by the method of Laemmli (1970) using 7.5% or 10% polyacrylamide resolving gels. Proteins resolved by SDS-PAGE were electrophoretically transferred to poly(vinylidene difluoride) by the method of Towbin et al. (1979). Membranes were incubated with rabbit polyclonal antibodies specific for either GLUT-4 (1 μg/ml) or vp165 (1 μg/ml), and immunoreactive proteins were detected using either ^{125}I-goat anti-rabbit IgG followed by autoradiography or HRP-conjugated goat anti-rabbit IgG (1:10,000) followed by ECL, respectively. For the quantitation of ECL signals different protein loadings were used on the gel for each sample to ensure that the response was linear.

RESULTS

Colocalisation of vp165 and GLUT4 in isolated vesicles

Previous biochemical studies have shown that there is a high degree of colocalisation between vp165 and GLUT4 in

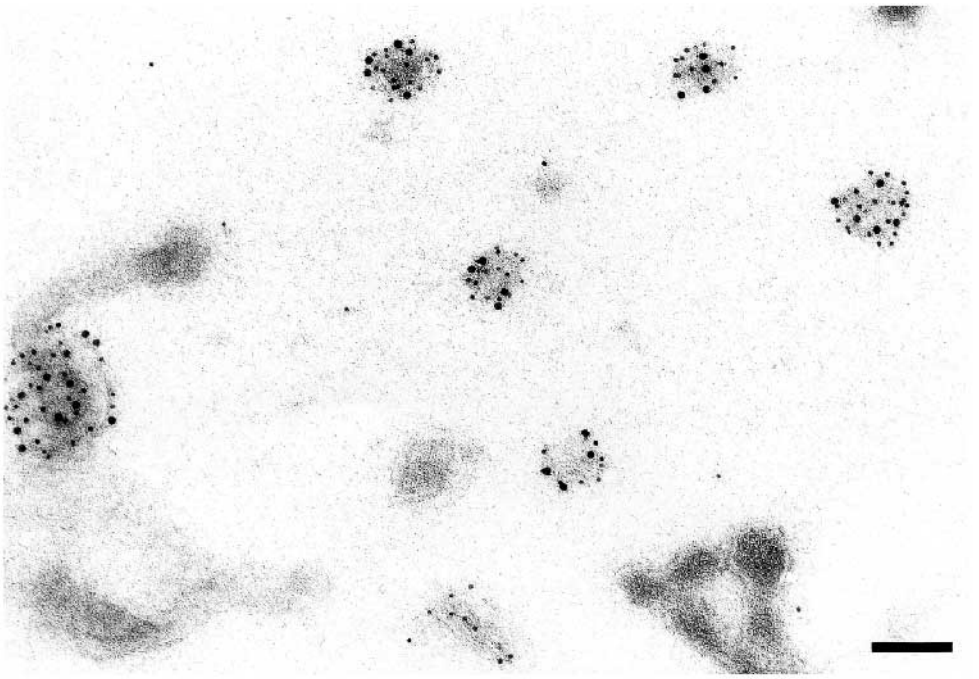


Fig. 1. Immuno-electron microscopy for vp165 and GLUT4 in rat adipocyte vesicles. Intracellular vesicles were prepared from non-stimulated rat adipocytes as described by Martin et al. (1996). Vesicles were absorbed to Formvar carbon-coated copper grids and double-labelled using antibodies specific for vp165 followed by Protein A-gold (10 nm), and GLUT4 followed by Protein A-gold (5 nm). Similar labelling was observed in vesicles isolated from 3T3-L1 adipocytes and rat heart (results not shown). Bar, 100 nm.

adipocytes (Mastick et al., 1994; Kandror and Pilch, 1994; Keller et al., 1995; Ross et al., 1996). We have recently used immuno-EM on whole mount vesicle preparations isolated from adipocytes to compare the distribution of GLUT4 with other markers in individual vesicles (Martin et al., 1996). Using this technique it is possible to both analyse the labelling, and detect variations in protein content across a uniform population of vesicles. Furthermore, it has been our experience that a much higher labelling efficiency can be achieved using isolated vesicles than using cryosections.

Vesicles were prepared from 3T3-L1 adipocytes, primary rat adipocytes (Fig. 1) and heart, and labelled for GLUT4 and vp165. The results from the analysis of labelling in adipocytes are shown in Table 1. As determined using single-labelling, the total labelled vesicle pool size was very similar for vp165 and GLUT4. Also, the amount of labelling for both antibodies was similar as illustrated by comparing the average number of gold particles per vesicle. This latter value is presented ± s.d. in order to demonstrate the range in the number of gold particles labelling individual vesicles. Using double-labelling the co-localisation between GLUT4 and vp165 was also determined (Table 1). In vesicles isolated from rat adipocytes there was a high degree of colocalisation, with 90.6% of the vp165-containing vesicles co-labelling for GLUT4, and 71.7% of the GLUT4-containing vesicles co-labelling with vp165 antibodies (Table 1 and Fig. 1). In 3T3-L1 adipocytes, 76.5% of the vp165-containing vesicles contained GLUT4 and 71.8% of the GLUT4-containing vesicles contained vp165. Thus, there is a high degree of colocalisation between GLUT4 and vp165 in adipocytes. Variations in the degree of colocalisation in rat adipocytes and 3T3-L1 adipocytes may be due to differences in the amounts of vp165 and GLUT4 expressed in these cells.

Vesicles were also prepared from total rat heart to compare the overlap in labelling in this tissue with that observed in adipocytes (Table 1). In these vesicles (which are predominantly derived from ventricular cardiomyocytes) vp165 and GLUT4 labelled 14% and 15% of the total vesicle pool, respectively. The average number of gold particles per vesicle was 10 for GLUT4 and 3 for vp165. There was again a very high degree of colocalisation between vp165 and GLUT4 in heart

Table 1. Analysis of vesicle labelling and colocalisation of vp165 and GLUT4, determined by immuno-EM

	Total vesicles labelled (%)		Labelling average* (gold particles/vesicle)		% Colocalisation	
	vp165	GLUT4	vp165	GLUT4	vp165 vesicles containing GLUT4	GLUT4 vesicles containing vp165
3T3-L1 adipocytes	16.9±2.5	18.1±2.8	4.0±3.8	2.7±2.6	76.5±5.6	71.8±7.0
Rat adipocytes	20.0±4.2	22.9±1.8	2.5±1.6	7.4±5.9	90.6±4.6	71.7±4.5
Rat heart	14	15	3	10	86	80

Intracellular vesicles were prepared from rat adipocytes, rat heart and 3T3-L1 adipocytes as described in Materials and Methods. Vesicles were single-labelled using antibodies specific for vp165 and GLUT4, followed by 10 nm Protein A-gold. For each protein in each cell type the percentage of the total vesicles labelled and the average number of gold particles per vesicle were determined. The degree of colocalisation between vp165 and GLUT4 was determined using double-labelling, as a function of the total population of either vp165 vesicles, or of GLUT4 vesicles, that contained the other marker. For rat heart a single preparation of vesicles was used. For rat adipocytes and 3T3-L1 adipocytes data represents the mean ± s.e.m. except * mean ± s.d., of 3 separate experiments. In each experiment 200-300 individual vesicles were quantified.

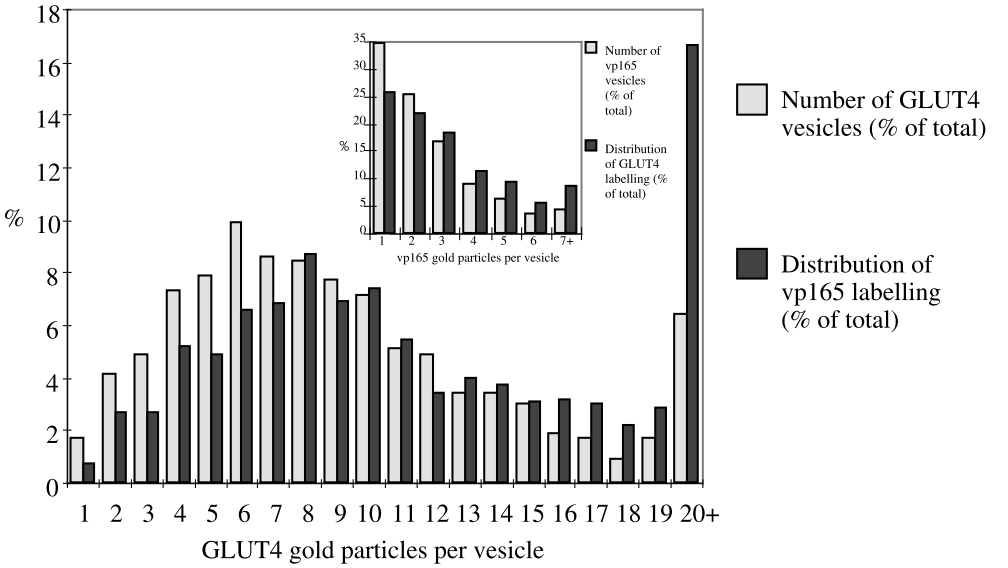


Fig. 2. Analysis of rat adipocyte vesicle double-labelling. Intracellular vesicles were isolated from rat adipocytes and double-labelled for GLUT4 and vp165. The number of GLUT4 gold particles and vp165 gold particles labelling each vesicle was determined and analysed. On the main graph the *x*-axis corresponds to the number of GLUT4 gold particles per individual vesicle. The *y*-axis corresponds to either the percentage of the total number of GLUT4 vesicles containing a specific number of GLUT4 gold, or to the percentage of the total amount of vp165 gold labelling which is present in those vesicles. The insert shows the equivalent analysis of the vp165 labelled vesicles. The total number of vesicles used in the analysis was 467. The total number of GLUT4 gold particles counted was >4,200, and the total number of vp165 gold particles was >1,200.

vesicles with 86% of the vp165-containing vesicles also containing GLUT4 and 80% of the GLUT4-containing vesicles co-labelling for vp165. Thus, these results were very similar to those obtained using vesicles from adipocytes.

In rat adipocyte vesicles the distribution of the number of gold particles per vesicle, summarised in Table 1, was analysed further (Fig. 2). The results in Fig. 2 demonstrate that most of the GLUT4 labelling is in vesicles that contain between 4-10 GLUT4 gold particles, with a peak at 6 gold. However, there are also vesicles which contain as few as 1-2 gold particles, and those that contain in excess of 20 gold particles. In an attempt to analyse the distribution of vp165 with respect to the distribution of GLUT4, the percentage of the total vp165 labelling present in the GLUT4 vesicles was also calculated. The distribution of vp165 mirrors that of GLUT4, however, the curve is offset slightly in favour of more vp165 in vesicles containing 6-10 GLUT4 gold particles, with a peak at 8 gold. Although the labelling was much lower for vp165 a similar result was obtained with the vp165 vesicles (inset, Fig. 2).

Endosome ablation

Studies of the intracellular distribution of GLUT4 following ablation of early endosomes has shown that GLUT4 is present in at least two biochemically distinct compartments. While one contains endosomal marker proteins such as the TfR and cellubrevin, the second compartment is largely devoid of these proteins (Livingstone et al., 1996; Martin et al., 1996). One aim of the present study was to determine whether vp165 is colocalised in this second compartment with GLUT4.

3T3-L1 adipocytes were incubated at 37°C for either 1 hour or 3 hours with the Tf/HRP conjugate prior to treatment with DAB ± peroxide. It has previously been shown that cells treated with DAB in the absence of peroxide are not effected by ablation (Livingstone et al., 1996) and therefore, this treatment was used as a control in all experiments. Two different time points were chosen to ensure that the TfR pathway was saturated with the Tf/HRP conjugate. In these experiments, ablation of the TfR was found to be >95% following a 3 hour uptake at 37°C (results not shown). The treated cells were fractionated and the amount of GLUT4 and vp165 in the intracellular membrane fraction was determined by immunoblotting (Fig. 3). The immunoblots were quantified by densitometry (Table 2). The amount of GLUT4 remaining in the intracellular membranes following ablation was 61% and 59% at 1 hour and 3 hours, respectively, and the amount of vp165 was 58% and 55% at 1 hour and 3 hours, respectively (Table 2).

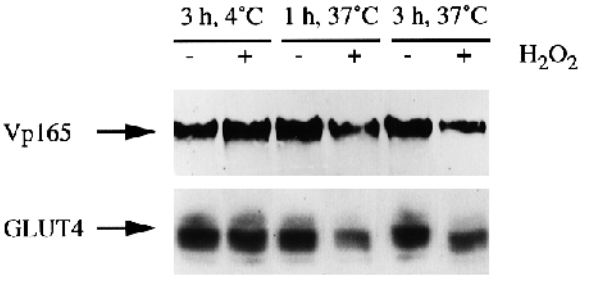


Fig. 3. Effect of Tf/HRP ablation on vp165 levels in 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated with a Tf/HRP conjugate for 1 hour or 3 hours at 4°C or 37°C as shown. Cells were then treated with DAB ± H_2O_2 prior to subcellular fractionation. The amount of vp165 and GLUT4 in the intracellular membrane fraction was determined by immunoblotting, and detected using ECL or ^{125}I-goat anti-rabbit IgG and autoradiography, respectively. Immunoblots were quantified by densitometry. The results of quantification are shown in Table 2.

Table 2. Effect of Tf/HRP ablation on intracellular GLUT-4 and vp165 levels

	% Marker remaining in the intracellular fraction					
	4°C, 1 hour		37°C, 1 hour		37°C, 3 hours	
H_2O_2	–	+	–	+	–	+
GLUT-4	100	105±8	105±9	61±7	102±4	59±5
vp165	100	110±6	105±11	58±7	109±4	55±10

3T3-L1 adipocytes were treated with Tf/HRP for either 1 hour or 3 hours at 37°C or for 3 hours at 4°C, after which the DAB cytochemistry reaction was performed in the presence (+) or absence (–) of H_2O_2. Cells were then homogenised and subjected to differential centrifugation to obtain intracellular membranes which were immunoblotted with antibodies specific for GLUT4 and vp165. The data are expressed as a percentage of the amount of immunoreactivity present in intracellular membranes isolated from cells that had been incubated with Tf/HRP for 3 hours at 4°C in the absence of H_2O_2. Data represent the mean ± s.e.m. of 3 experiments.

Following incubation with the Tf/HRP conjugate for 3 hours at 4°C and subsequent treatment with DAB/peroxide there was no significant ablation of either GLUT4 or vp165 (Fig. 3) indicating that ablation of these proteins at 37°C was dependent on the recycling of the Tf/HRP conjugate through the endosomal pathway. Together with the results of the vesicle labelling, these results strongly suggest that vp165 is highly colocalised with GLUT4, both in endosomes and in the separate, post-endocytic compartment.

Cell and tissue expression of GLUT4 and vp165

Prior to immunocytochemistry the expression of vp165 and GLUT4 was compared in heart, skeletal muscle, adipocytes, 3T3-L1 adipocytes and 3T3-L1 fibroblasts. As found in other studies (Kandror and Pilch, 1994; Keller et al., 1995) both proteins were strongly expressed in all three tissues and in 3T3-L1 adipocytes (Fig. 4). However, in agreement with previous results (Ross et al., 1996) only vp165, not GLUT4, was expressed in 3T3-L1 fibroblasts. The results of tissue immunoblotting suggested that heart tissue expressed high levels of vp165 protein. To further characterise the expression of vp165 in heart, cardiac muscle was separated into three different regions: atrium, ventricle and papillary muscle. Immunoblots for GLUT4 and vp165 clearly show that both proteins are expressed in all three regions (Fig. 4). However, GLUT4, in agreement with previous studies (Slot et al., 1997), is more abundant in the atrium than in the ventricle, and vp165 is more abundant in the atrium and in the papillary muscle than in the ventricle.

Intracellular localisation of vp165 in atrial cardiomyocytes

The intracellular localisation of vp165 was compared to that of GLUT4 in tissue sections obtained from the atrium. The atrium was selected for these studies because we have previously shown that atrial cardiomyocytes contain two separate regulated GLUT4 compartments (Slot et al., 1997). One compartment, which contains approximately 40% of the total GLUT4 in these cells, resembles the well-described insulin-regulatable T-V elements found in brown adipose tissue and in ventricular cardiomyocytes (Slot et al., 1991a,b). The remaining GLUT4 in the atrial cardiomyocyte is targeted to the ANF-containing secretory granules (Slot et al., 1997). This compartment is functionally distinct from the T-V elements as ANF, an anti-hypertensive hormone, is secreted in response to various stimuli (De Bold, 1985; Ruskoaho, 1992) including stretch, hypoxia, and thrombin (Klapper et al., 1996), but serum levels are not elevated in response to insulin (Slot et al., 1997). Thus, we felt that colocalisation experiments in this cell type using antibodies specific for GLUT4 and vp165 would provide a rigorous test of the similarity in targeting between these two proteins.

By immunofluorescence, in longitudinal sections of rat atrium, GLUT4 was concentrated in a perinuclear location (Fig. 5A), where at higher magnification it was clearly labelling around granules. This labelling pattern was very similar to that of ANF (Fig. 5C). In addition GLUT4 labelling was distributed as punctate labelling throughout the cells, presenting a striated labelling pattern in areas where the cells had been cut longitudinally (Fig. 5A). This pattern corresponds to the sarcomeric Z-line distribution described previously for GLUT4 (Slot et al., 1991a). In contrast, strong perinuclear labelling was not observed for vp165 (Fig. 5B), but a prominent dispersed punctate and striated labelling pattern, as described above for GLUT4, was. These results suggested differences in the distribution of these two proteins in atrial cardiomyocytes.

Using electron microscopy, the GLUT4 subcellular distribution in atrium reported in this study was very similar to that resolved in previous studies (Slot et al., 1997). The amount of vp165 labelling in the sections was much lower than the labelling for GLUT4. However the labelling was highly

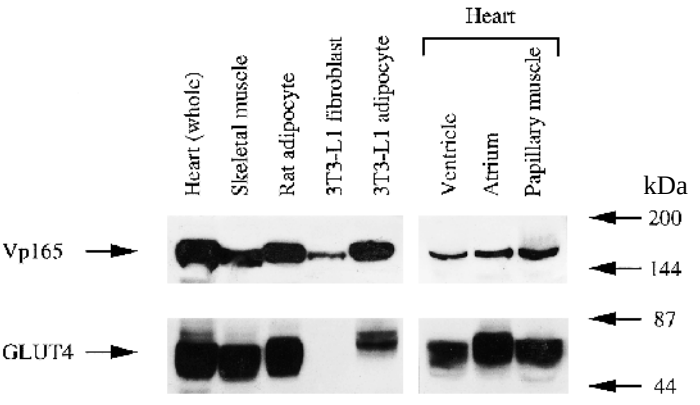


Fig. 4. Expression of GLUT4 and vp165 in heart, skeletal muscle, adipocytes, 3T3-L1 fibroblasts and 3T3-L1 adipocytes. Total membrane fractions were prepared from various insulin-regulatable rat tissues and from 3T3-L1 adipocytes and fibroblasts. Samples of each tissue (50 μg protein) were resolved by SDS-PAGE using 7.5% polyacrylamide gels, and immunoblotted for both GLUT4 and vp165. Immunolabelled proteins were detected using ECL.

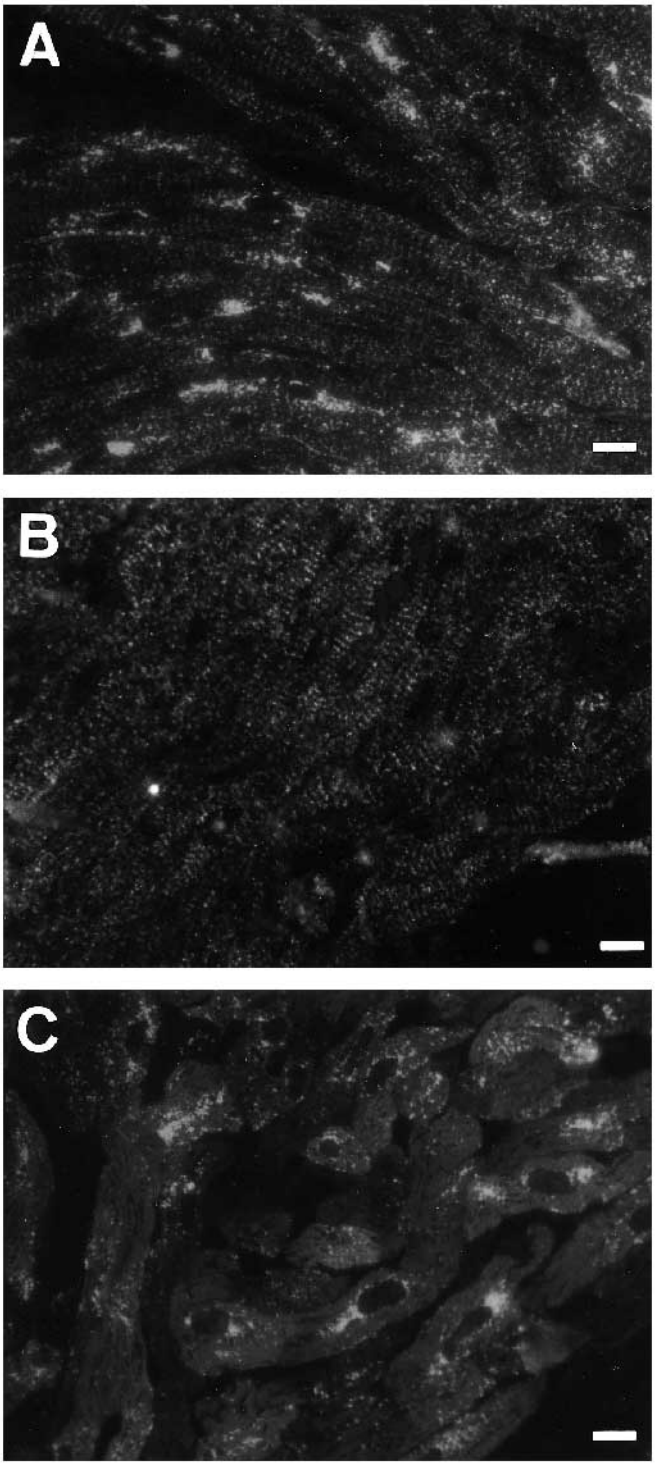


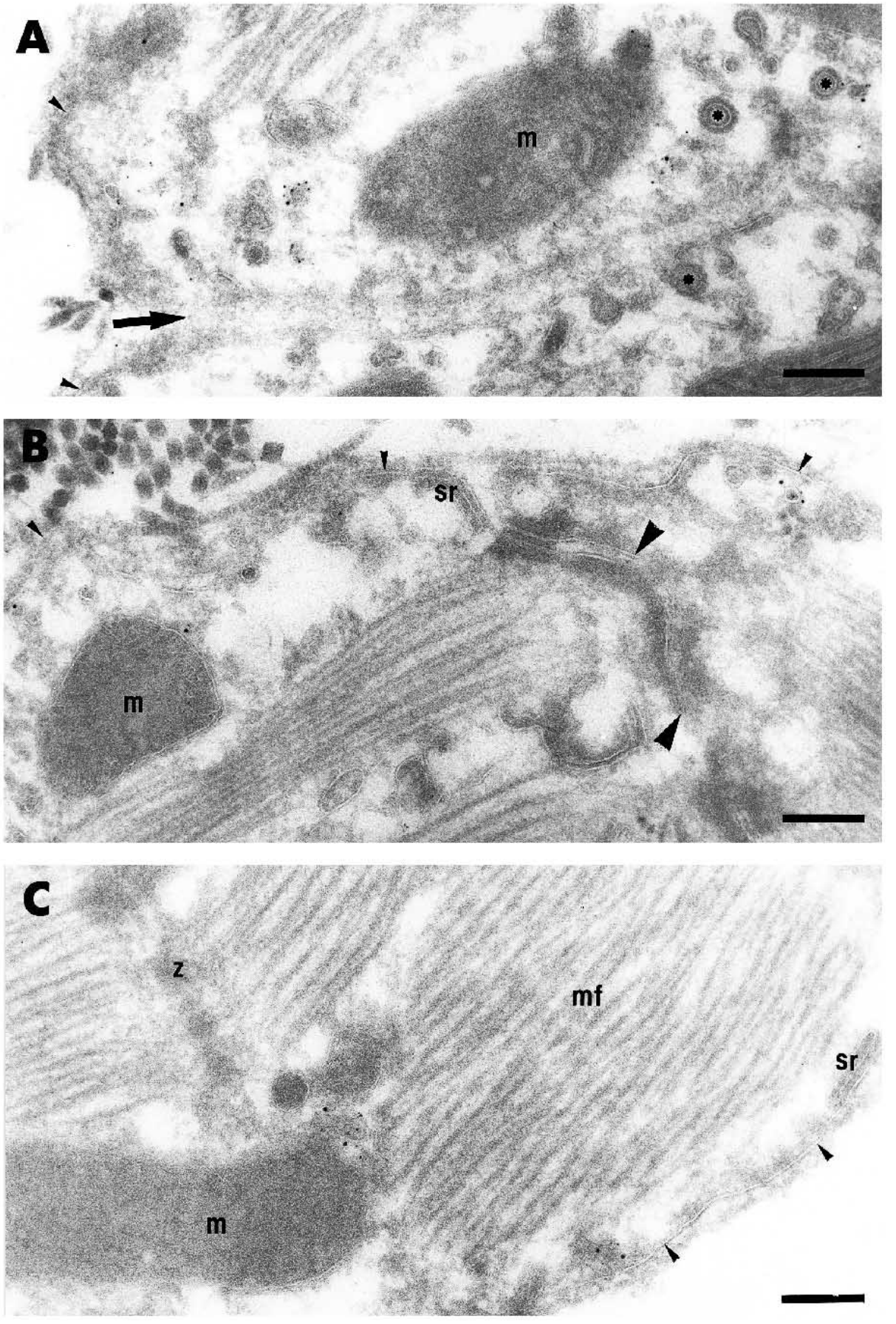
Fig. 5. Immunofluorescence labelling of vp165, GLUT4 and ANF in atrium. Cryosections of atrium were labelled for either GLUT4 (A), vp165 (B), or ANF (C), and the distribution determined by immunofluorescence. ANF labelling (C) was predominantly perinuclear. The labelling for GLUT4 was both perinuclear (A), and distributed throughout the cells. In regions were the atrium had been cut in a longitudinal plane, the GLUT4 labelling appeared to be periodic. This striated labelling pattern has been previously described (Slot et al., 1991a, 1997), and shown to follow the sarcomeric periodicity. Labelling for vp165 (B) was much lower than either GLUT4 or ANF. It was distributed throughout the cells although in regions where the atrium was cut longitudinally there was periodicity to the labelling pattern, similar to GLUT4. There was no concentration of vp165 labelling in the perinuclear regions of the cells. Bars, 10 μm.

specific. No specific labelling was seen in control sections labelled using irrelevant IgG (results not shown). T-V elements containing both GLUT4 and vp165 were present scattered throughout the cell (Figs 6, 7), in the TGR (Fig. 8) and occasionally on the trans-Golgi cisternae (Fig. 8). There was no significant labelling of the plasma membrane because tissue was obtained from fasted, non-stimulated animals (Fig. 6). In contrast to recent studies in skeletal muscle (Wang et al., 1996) there was also no significant labelling of the sarcoplasmic reticulum. Around the myofibres the labelling was often, but not exclusively, at the level of the Z-lines (Fig. 7). T-V elements containing GLUT4 and vp165 were also observed close to t-tubule membranes (Fig. 6A), and appeared to follow these specialised plasma membrane domains towards the interior of the cell. Additionally, a large proportion of the GLUT4 labelling was found on the membrane of the ANF granules (Figs 7, 8). These granules were confirmed as ANF granules using an antibody against the N terminus of the pro-ANF polypeptide (results not shown). Most strikingly, however, in contrast to the density of GLUT4, the ANF granule membranes were largely devoid of vp165 labelling (Figs 7, 8).

To determine the relative distribution of vp165 and GLUT4 between the T-V elements (including the TGR) and the ANF granules the labelling was quantified in sections of atrial cardiomyocytes (Table 3). Over 50% of GLUT4 was found on the ANF granule membrane, whereas there was less than 5% of vp165 associated with this structure. In contrast 95% of vp165 was present in the T-V elements. Although vp165 was not found in ANF granules, it was notable that vp165 positive vesicles were occasionally observed in close proximity to the ANF granules (Fig. 8A).

We have previously suggested that GLUT4 enters the ANF granules as they form in the TGR (Slot et al., 1997). Therefore, the absence of vp165 labelling in the granules could simply reflect a difference in the absolute amounts of GLUT4 and vp165 in the TGR. The amount of GLUT4 relative to vp165

Fig. 6. Immuno-EM of GLUT4 and vp165 labelling in regions of atrial cardiomyocytes proximal to the plasma membrane. Cryosections of atrial cardiomyocytes are immunogold labelled for vp165 (10 nm gold) and GLUT4 (5 nm gold). All three fields show labelling adjacent to plasma membrane domains. In A the labelling is close to a t-tubule, and appears to follow the path of the t-tubule towards the interior of the cell. A large number of clathrin-coated, predominantly unlabelled, vesicles are present around, and budding from, the t-tubule membrane. In contrast to the t-tubule, double-labelled T-V elements were not common in proximity to the intercalated discs (B) between two adjacent cardiomyocytes. As both intercalated discs and easily identifiable t-tubules were relatively rare in atrial myocytes, the double-labelled T-V elements were predominantly observed close to the sarcolemma (C). There was no labelling of the plasma membrane or sarcoplasmic reticulum. Large arrow, t-tubule; large arrowheads, intercalated disc; *, clathrin-coated vesicles; small arrowheads, plasma membrane; m, mitochondrion; mf, myofibres; z, Z-line; sr, sarcoplasmic reticulum. Bar, 200 nm.


A
m
B
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m

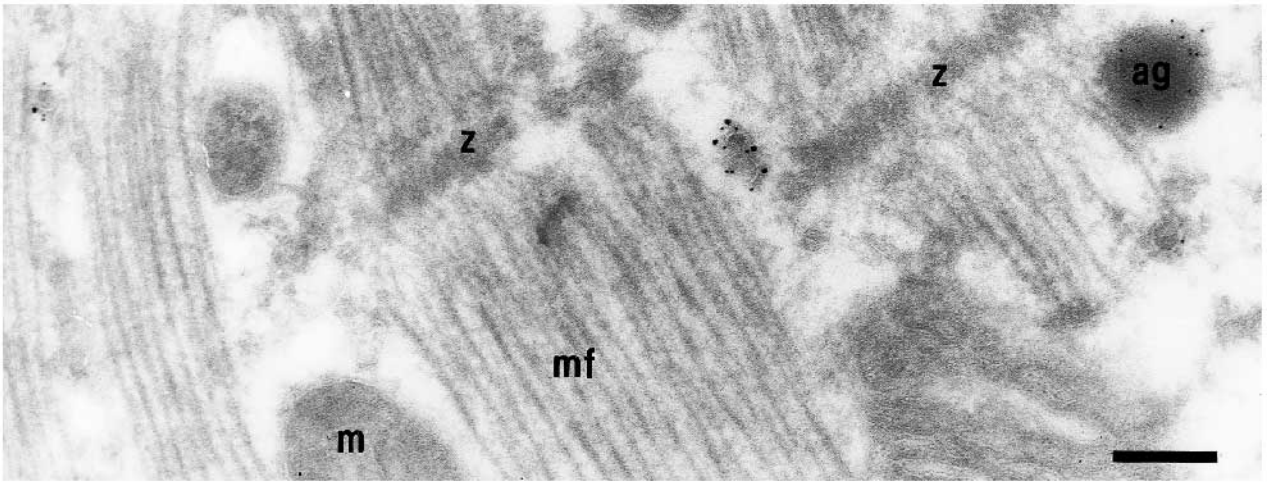


Fig. 7. Immuno-EM of GLUT4 and vp165 labelling in dispersed T-V elements of the atrial cardiomyocyte. Cryosections were double-labelled for vp165 (10 nm gold) and GLUT4 (5 nm gold). Labelled T-V elements were distributed throughout the atrial cardiomyocyte, however, they were often, but not exclusively located in parallel to the Z-line of the myofibre. ANF granules (ag) labelled for GLUT4 were also distributed throughout the cell, although they were most concentrated in the perinuclear, Golgi-enriched areas (see Fig. 8). These secretory granules did not label for vp165, although occasionally vp165 labelled T-V elements were in very close proximity to them. Bar, 200 nm.

Table 3. Relative distribution of GLUT4 and vp165 in rat atrial cardiomyocytes

	Gold labelling (%)	
	GLUT4	vp165
T-V elements/TGR	41.6±5.0	94.6±1.5
ANF granules	58.2±5.0	3.8±0.3
Plasma membrane	0.2±0.1	1.6±1.0

Gold particles were counted randomly in longitudinally cut sections of basal atrial cardiomyocytes. Over these regions the cells were transversed at an angle of approximately 90° to the myofibres. Three sections from two different atria were counted and averaged. In the same area random points were projected on the sections. These points were assigned to myocyte structures as if they were gold particles. Approximately 90% of the random points were found over unlabelled structures. Over 95% of the GLUT4 labelling was found on the structures included in the table, together with 75% of the vp165 labelling. The remaining vp165 labelling was predominantly over mitochondria and myofibres and was considered non-specific. The Golgi/TGR and T-V elements were combined for comparison with the ANF granules. For GLUT4 between 300-600 gold particles, and for vp165 between 40-60 gold particles, were quantified per section, respectively. Results are expressed as the mean ± s.e.m.

Table 4. Ratio of GLUT4 to vp165 in atrial subcellular compartments

Subcellular compartment	GLUT4/vp165
Golgi/TGR	15.3±6.3
T-V elements	2.7±0.5
ANF granules	50-300

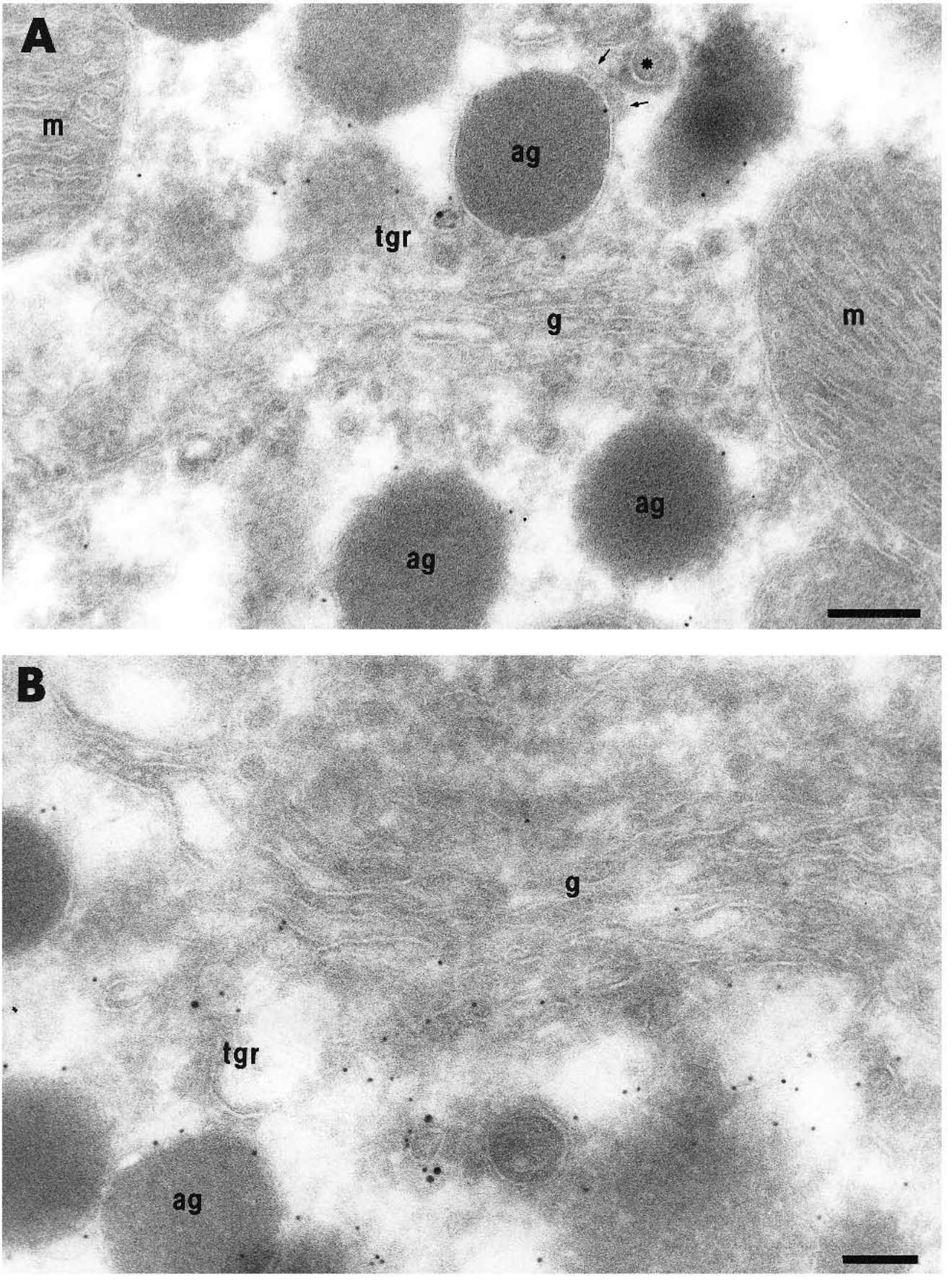
The gold labelling for GLUT4 and vp165 was counted in each of the subcellular compartments shown above randomly encountered in double-labelled sections. For each compartment the relative number of GLUT4 gold particles per vp165 gold particle was calculated. Results shown are the average number of GLUT4 per vp165. Three separate sections were quantified. A total of >500 gold particles were counted for GLUT4 in each compartment. Results are expressed as mean ± s.e.m. except for ANF granules. Due to the very low amount of vp165 in the ANF granules relative to the GLUT4 labelling this ratio was not averaged, and the range is shown.

was therefore determined for the TGR and compared to dispersed T-V elements. This analysis clearly demonstrates a marked excess of GLUT4 relative to vp165 in the TGR (Table 4) when compared to the relative levels in the T-V elements. However, it still does not fully explain the absence of vp165 from the ANF granules, as the ratio of GLUT4 to vp165 in the granules (Table 4) is much higher than in the TGR.

DISCUSSION

Evidence is now accumulating to suggest that GLUT4 is targeted to multiple intracellular compartments in adipocytes (Verhey et al., 1995; Martin et al., 1996). Determining the nature of these compartments will be fundamental in defining the mechanism by which GLUT4 is translocated to the cell surface in response to insulin. It has been shown that immunoprecipitation of vesicles containing GLUT4 results in the co-isolation of vp165 (Kandror and Pilch, 1994; Mastick et al., 1994), suggesting that these two proteins have a high degree of colocalisation. In the present study, immuno-EM on vesicles isolated from adipocytes and cardiomyocytes confirmed that the colocalisation between GLUT4 and vp165 is striking. Furthermore, using an endosomal ablation technique following uptake

Fig. 8. Immuno-EM of GLUT4 and vp165 in TGR and ANF granules. Immunogold labelling of vp165 (10 nm gold) and GLUT4 (5 nm gold) in the Golgi region of atrial cardiomyocytes. Adjacent to the nuclei of the atrial cardiomyocytes are regions densely packed with ANF granules and enriched in Golgi (g). In these regions GLUT4 labelling is present on the ANF granules, in the TGR (tgr) and in *trans*-Golgi cisternae (A,B), while the vp165 labelling is predominantly present in the TGR (A,B). There is no vp165 on the ANF granules. In the region of the Golgi, T-V elements labelled for vp165 were observed in very close proximity to the ANF granules (A). However, these T-V elements did not appear to be coated, and were not close to the patches of clathrin coat (small arrows) often seen on the ANF granule. Bar, 100 nm.


A
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of a Tf/HRP conjugate we show that vp165 is distributed among distinct intracellular compartments in a similar way to GLUT4 in 3T3-L1 adipocytes. In fact, of all markers that we have examined thus far using these techniques, vp165 shows the highest degree of colocalisation with GLUT4. We also demonstrate that vp165 and GLUT4 colocalise almost completely within a tubulo-vesicular compartment in atrial cardiomyocytes similar to that previously identified as housing GLUT4 in brown adipose tissue and ventricular cardio-myocytes (Slot et al., 1991a,b). Thus, overall these studies suggest that these two proteins are sorted in a highly analogous manner when expressed together in both muscle and adipose tissue. This may be due to similar targeting properties and may also be facilitated by a close association between the two proteins. Work is currently under way to determine if this is the case. It is interesting to note however, that vp165 has a broader tissue distribution than GLUT4, and is expressed in all major tissues, except in liver, where the amount is low (Keller et al., 1995). The function of vp165 in any tissue is not yet known. However, preliminary studies have indicated that in other cell types, in which glucose transport is not acutely regulated by insulin, vp165 has a different localisation to that described in the present study (J. Slot, unpublished observations).

Despite the high degree of overlap between GLUT4 and vp165 we present evidence that the intracellular distribution of these proteins is not identical. In the atrial cardiomyocyte, in contrast to GLUT4, vp165 was not found in the ANF secretory granules. This is unlikely to be due to a low detection or labelling density for vp165, as the antibodies clearly labelled vp165 when it was present in T-V elements. We have previously suggested that GLUT4 enters this secretory compartment at the level of the TGR, as the secretory granule is budding from the Golgi (Slot et al., 1997). As it is known that *trans*-Golgi reticular GLUT4 is largely derived from a recycling pathway and not from the biosynthetic route (Slot et al., 1997) this result suggests differences in the subcellular trafficking pathways or retention times between GLUT4 and vp165 in the TGR.

The presence of vp165 in the TGR was morphologically demonstrated in immunolabelled cryosections (Fig. 8), and indirectly confirmed by the colocalisation vp165 with the γ-adaptin subunit of the TGR adaptor complex, AP-1, in vesicles from adipocytes (S. Martin and D. E. James, unpublished data). However, the absence of vp165 from the granules could partly reflect the fact that the TGR contains relatively little vp165 compared to the T-V elements (Table 4). Another intriguing possibility is suggested by the presence of AP-1 positive patches of clathrin coat on the ANF granules (Slot et al., 1997) which could provide the basis for an exit pathway for membrane proteins. However, there is little structural homology between vp165 and GLUT4 and so it is highly likely that they may exhibit differences in targeting in different cell types. Numerous studies have been performed attempting to dissect linear targeting motifs in GLUT4, that encode for its intracellular sequestration (Piper et al., 1993: Corvera et al., 1994; Verhey and Birnbaum, 1994; Marsh et al., 1995; Verhey et al., 1995). Now with the identification of another protein that appears to be targeted very similarly to GLUT4 it will be possible to compare and contrast these motifs between the two proteins. It has been proposed that GLUT4 contains at least two separate targeting domains. One is in the C-terminal cytoplasmic tail and involves a dileucine motif similar to that described in numerous other recycling proteins (Shin et al., 1991; Johnson and Kornfeld, 1992; Chen et al., 1993; Pieters et al., 1993; Aiken et al., 1994; Bremnes et al., 1994; Pond et al., 1995; Dietrich et al., 1996; Dittrich et al., 1996). In addition, it has been suggested that there may be a second, separate targeting motif in this tail (Verhey et al., 1995). Although vp165 has only a single cytoplasmic domain it contains multiple dileucine motifs and a highly acidic region (Keller et al., 1995) similar to that found at the extreme C terminus of GLUT4 (James et al., 1989). Hence, it will be of interest to determine if these motifs subserve a common function in both proteins. The N-terminal cytoplasmic tail of GLUT4 has also been suggested to play an important role in the molecular targeting of the protein (Piper et al., 1992, 1993; Marsh et al., 1995). This region contains a FQQI motif similar to tyrosine-based motifs found in other recycling proteins (reviewed by Trowbridge et al., 1993). It is of particular interest that there is a high degree of homology between this region of GLUT4 and the N-terminal of GAD65, the region suggested to encode for targeting information that allows the latter protein to associate with secretory granules (Shi et al., 1994; Solimena et al., 1994). Considering that this domain is not conserved in vp165, this may explain why GLUT4 but not vp165 is targeted to secretory granules in atrial cardiomyocytes.

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