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GLUT4 in cultured skeletal myotubes is segregated from the transferrin receptor and stored in vesicles associated with the TGN

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SUMMARY

There is little consensus on the nature of the storage compartment of the glucose transporter GLUT4, in non-stimulated cells of muscle and fat. More specifically, it is not known whether GLUT4 is localized to unique, specialized intracellular storage vesicles, or to vesicles that are part of the constitutive endosomal-lysosomal pathway. To address this question, we have investigated the localization of the endogenous GLUT4 in non-stimulated skeletal myotubes from the cell line C2, by immunofluorescence and immunoelectron microscopy. We have used a panel of antibodies to markers of the Golgi complex (α mannosidase II and giantin), of the *trans*-Golgi network (TGN38), of lysosomes (lgp110), and of early and late endosomes (transferrin receptor and mannose-6-phosphate receptor, respectively), to define the position of their subcellular compartments. By immunofluorescence, GLUT4 appears concentrated in the core of the myotubes. It is primarily found around the nuclei, in a pattern suggesting an association with the Golgi complex, which is further supported by colocalization with giantin and by immunogold electron microscopy. GLUT4 appears to be in the *trans*-most cisternae of the Golgi complex and in vesicles just beyond, i.e. in the structures that constitute the *trans*-Golgi network (TGN). In myotubes treated with brefeldin A, the immunofluorescence pattern of GLUT4 is modified, but it differs from both Golgi complex markers and TGN38. Instead, it resembles the pattern of the transferrin receptor, which forms long tubules. In untreated cells, double staining for GLUT4 and transferrin receptor by immunofluorescence shows similar but distinct patterns. Immunoelectron microscopy localizes transferrin receptor, detected by immunoperoxidase, to large vesicles, presumably endosomes, very close to the GLUT4-containing tubulovesicular elements. In brefeldin A-treated cells, a network of tubules of ~70 nm diameter, studded with varicosities, stains for both GLUT4 and transferrin receptor, suggesting that brefeldin A has caused fusion of the transferrin receptor and GLUT4-containing compartments. The results suggest that GLUT4 storage vesicles constitute a specialized compartment that is either a subset of the TGN, or is very closely linked to it. The link between GLUT4 vesicles and transferrin receptor containing endosomes, as revealed by brefeldin A, may be important for GLUT4 translocation in response to muscle stimulation.

Key words: Muscle, GLUT4, Golgi complex

INTRODUCTION

Stimulation by insulin results, in muscle and adipose tissue, in a large, acute increase in glucose uptake, which is mediated by the facilitative glucose transporter GLUT4, one member in a family of altogether six glucose transporter proteins (Bell et al., 1993). Upon insulin stimulation, GLUT4 appears to translocate from an intracellular storage compartment to the plasma membrane. This mechanism is supported by subcellular fractionation analysis of both adipose cells and muscle (for reviews see Birnbaum, 1992; Holman and Cushman, 1994) and by the detailed quantitative immunogold analysis of GLUT4 localization in rat adipose tissue (Slot et al., 1991). In skeletal muscle, stimulation by contractions also induces translocation of GLUT4 to the surface of the cell (Douen et al., 1989; Ploug et al., 1993).

Of the many questions that remain unanswered, to do with localization and trafficking of GLUT4 (James et al., 1994), one of the most debated is whether GLUT4 storage vesicles, in unstimulated cells, represent a separate, specific class of vesicles. Several other membrane proteins appear to be translocated to the plasma membrane following insulin stimulation, suggesting that they may be in the same vesicles. The transferrin receptor (TfR) is one of these proteins (Davis et al., 1986; Tanner and Lienhard, 1987), and its well-described endocytosis via coated vesicles and endosomes and rapid recycling to the plasma membrane (Klausner et al., 1983; Dautry-Varsat et al., 1983; Yamashiro et al., 1984) present many features in common with the trafficking of GLUT4. Several studies have addressed the possible colocalization of GLUT4 and TfR, without reaching a consensus: there have been reports that the two colocalize totally (Tanner and

Lienhard, 1989), partially (Hudson et al., 1992, 1993; Laurie et al., 1993; Martin et al., 1994), or not at all (Herman et al., 1994; Aledo et al., 1995).

The lack of consensus may result from differences in the various cell types used in these studies, several of which were carried out on non-insulin-responsive cells transfected to express GLUT4, and others on different types of fat cells. Despite the predominant role of muscle in the pathophysiology of non-insulin-dependent diabetes (De Fronzo, 1992), little of this work has been carried out on muscle cells. In fact, it was believed for years that only the L6 muscle cell line expresses and translocates GLUT4 and that widely used muscle cell lines such as C2 and BC_3H_1 do not express detectable amounts of GLUT4 (Sargeant et al., 1993). Studies of GLUT4 localization in muscle, in vivo and in vitro, may help to explain the features of glucose transport that are common to insulin-responsive tissues, and those that are specific to muscle cells, whose geometry and subcellular architecture differ considerably from those of fat cells.

The goal of the present work is to identify the GLUT4 storage compartment in C2 myotubes and to determine its relationship to the subcellular organelles involved in the endosomal-lysosomal pathway. Using immunofluorescence and immunogold electron microscopy, we observe that the majority of GLUT4 is stored in vesicles that are intimately associated with the Golgi complex and do not contain TfR. We use the fungal metabolite brefeldin A (BfA) to further analyze these vesicles and highlight their functional connections to other organelles. We find that GLUT4 is dramatically redistributed in BfA-treated cells. Surprisingly, however, its pattern is now distinct from that of several markers of the Golgi complex and, instead, it colocalizes with the TfR. These results suggest that GLUT4 is stored in a specific subcompartment of the TGN, and has a functional, dynamic connection with the TfR-containing endosomes.

MATERIALS AND METHODS

Antibodies and reagents

Primary antibodies are listed in Table 1. Anti-mouse transferrin receptor (TfR) was a gift from Dr Jayne Lesley (Salk Institute); anti-lgp110 from Dr Bruce Granger (Montana State University); anti-TGN38 from Dr Paul Luzio (Medical Research Council, Cambridge); anti-mannose-6-phosphate receptor/type II insulin-like growth factor receptor (M6PR) from Dr Peter Nissley (National Cancer Institute, NIH); anti-α-mannosidase II from Dr Kelley Moremen (University of Georgia); and anti-giantin from Dr Adam Linstedt (Carnegie Mellon Institute). Fluorescein- and rhodamine-conjugated secondary antibodies were purchased from Organon-Teknika (Malvern, PA) and biotinylated secondary antibodies and fluorescein- and Texas red-conjugated avidin were from Vector Laboratories (Burlingame, CA). Nanogold-conjugated anti-rabbit Fab fragments and silver enhancement kits (HQ) were from Nanoprobes Inc. (Stony Brook, NY). Brefeldin A was obtained from Epicentre Technologies (Madison, WI).

Cell cultures

C2 cultures (Yaffe and Saxel, 1977) were grown and maintained as described (Ralston and Hall, 1989), except that fusion medium contained 4% horse serum. Once in fusion medium, half of the medium was replaced daily. Myoblasts were plated on glass coverslips coated with a layer of carbon, followed by 0.1% gelatin (Daniels, 1990). Before fixation for staining, the medium was replaced with serum-free medium for 2 hours. In some experiments, BfA (1 mg/ml in ethanol) was added to the cultures to a final concentration of 10 μg/ml, 30 minutes before fixation.

Immunofluorescence

Cells were rinsed in phosphate-buffered saline (PBS) and fixed for 15 minutes with 4% formaldehyde from a 16% stock (Electron Microscopy Sciences, Ft. Washington, PA) diluted in calcium- and magnesium-free PBS. After blocking for 40 minutes in PBS containing 3% goat serum, 1% bovine serum albumin and 0.02% saponin (all from Sigma, St Louis, MO), cells were stained for 2 hours with the primary antibody in the same solution, rinsed three times with PBS containing 0.02% saponin, stained for 1 hour with the secondary antibody, washed again, and mounted in Vectashield (Vector Laboratories). For biotinylated secondary antibodies, there was an additional incubation for 20 minutes with Texas red or fluorescein-conjugated avidin in 20 mM Tris-HCl, 150 mM NaCl, 0.02% saponin at pH 8.5. Staining was observed with a Zeiss Axioskop fluorescence microscope or with a Leitz DMRD, both fitted with narrow bandpass filters. Confocal images were obtained on a Zeiss LSM 410 confocal microscope fitted with 488 nm and 568 nm krypton-argon lines, using a ×100 (NA 1.4) lens and zoom 2.5. The images were printed in Photoshop 3.0 on a Macintosh.

The results presented here are representative of several experiments. Staining with single markers was repeated at least three times; TfR-M6PR double staining, twice; and TfR-GLUT4 double staining, four times.

Immunogold electron microscopy

Cultures were rinsed with warm PBS and fixed with 4% formaldehyde at 37°C for 30 minutes, then at room temperature for 30 minutes, and finally at 4°C overnight. For cultures treated with BfA, the reagent was also added to the fixative. Permeabilization and staining were as described for immunofluorescence, except that the secondary antibody for GLUT4 was anti-rabbit Fab fragments conjugated to a 1.4 nm gold cluster and for TfR a biotinylated anti-rat IgG followed by ABC peroxidase (Vector Laboratories). Peroxidase was developed for 10 to 30 minutes with diaminobenzidine (DAB; 0.5 mg/ml) and H_2O_2 (0.5 μl of 30% stock per ml of DAB). Cells were postfixed with 1% glutaraldehyde for 15 minutes and silver-enhanced according to

Table 1. Antibodies used in this work

Antigen	Compartment	Name of ab	Species and type*	Reference
GLUT4	?	P-1	Rabbit pc	Ploug et al. (1990)
TfR	Early endosomes	2I7 217	Rat mc	Lesley et al. (1989)
lgp 110	Lysosomes	GL2A7Kn	Mouse mc	Granger et al. (1990)
M6PR	Late endosomes/TGN	3637	Rabbit pc	Kiess et al. (1987)
TGN38	TGN		Rabbit pc	Luzio et al. (1990)
α mannosidase II	Golgi cisternae		Rabbit pc	Moremen and Touster (1985)
Giantin	Golgi cisternae		Mouse mc	Linstedt and Hauri (1993)

*pc, polyclonal antibody; mc, monoclonal antibody.

the manufacturer's recommendations. Samples were osmicated with 0.2% OsO_4, en bloc mordanted with uranyl acetate, dehydrated in graded ethanol and embedded in Epon. Glass coverslips were separated from the resin block by brief immersion in liquid nitrogen, and fragments of the blocks were sectioned en face. Sections of 70 and 200 nm, unstained, were observed with a Jeol 1200 microscope at 60 kV. The results presented here are representative of four separate experiments with double staining and additional experiments with single staining. In two experiments, duplicate coverslips were silver-enhanced for different times. Longer silver enhancement produces larger grains that are easier to visualize but tend to increase background and to hide underlying membranes. Controls with omission of the first or of the secondary antibody were included in each experiment.

RESULTS

Immunofluorescence localization of endogenous GLUT4 in muscle cultures

Fig. 1 shows permeabilized cultures of the mouse muscle cell line C2 stained with the anti-GLUT4 antibody P-1 at four developmental stages and observed by immunofluorescence.

Myoblasts (Fig. 1a, arrow) have low levels of GLUT4 (confirmed by immunoblotting, results not shown). Some show a staining of large aggregates, often at both poles of the nucleus, whereas most (small arrows) only show a fine punctate staining. In myotubes, the staining encircles the nuclei with an interrupted ring, and forms additional aggregates, often aligned along the axes of the myotubes. The position of these aggregates is not fixed in relation to the nuclei and their number and intensity varies along the myotubes, thus creating some variability in the pattern. As myotubes mature (from 1 to 6 days in fusion medium, Fig. 1b-d), the basic pattern remains unchanged. Most of the staining is concentrated in the central core of the myotubes. Only in the most mature cultures (Fig. 1d) can a fine punctate staining be detected towards and occasionally at the edge of the cell, which is marked by small arrowheads. Thus, GLUT4, in myotubes, appears to be efficiently excluded from the cell membrane, as has also been shown in other cells.

Although we only show results obtained with C2 myotubes, we also find GLUT4 in Sol 8 myotubes, in differentiated cultures of the non-fusing muscle cell line BC_3H1, and in rat primary myotubes. The intensity and pattern of staining are roughly similar in all and comparable to what we observe in L6 myotubes. Thus GLUT4 is expressed as part of the normal myogenic development in all muscle cells.

GLUT4 is stored in the Golgi complex area

In an attempt to identify the GLUT4 storage compartment, we used a panel of antibodies, described in Table 1, that recognize markers of the Golgi complex, of the *trans*-Golgi network (TGN), of early and late endosomes, and of lysosomes. Since we have observed that TfR and GLUT4 are upregulated during development of C2 cultures, whereas α mannosidase II, M6PR and TGN38 are downregulated, all the experiments presented are with myotubes that have been in fusion medium for 3 to 4 days, by which time all markers can readily be detected. The staining patterns are presented in Fig. 2, with each panel showing a single representative myotube. GLUT4 (GT4) and lgp110 panels show the same myotube double-stained with the two antibodies. All other antibodies were applied individually. To some degree, all markers show some concentration around the nuclei but the GLUT4 resembles mannosidase (man) the most in pattern and location. Both form interrupted rings around the nuclei (small arrowheads) and present aggregates between the nuclei. In both cases, the staining is concentrated in the central core of the myotubes. Staining for TGN38 also shows large aggregates around and between nuclei, but superimposed on a finer punctate staining that covers the whole myotube. In contrast, staining for M6PR and lgp 110 gives a finer punctate staining, more scattered throughout the cell. The differences between distribution of GLUT4 and lgp110 are clear from the examination of the myotube shown here: there is little if any overlap in the two patterns and lgp110 staining is extensive in areas near the plasma membrane where there is no GLUT4 (large arrowhead in lgp110 panel). TfR is the only marker that shows a distinct staining of the plasma membrane (arrow). In the photograph shown here, focus was on the fraction of TfR that appears in the perinuclear area. The pattern appears finer, more continuous than that of GLUT4.

In order to further explore the link between GLUT4 and the Golgi complex, we double-stained C2 myotubes for GLUT4 and for giantin, a protein believed to be involved in the

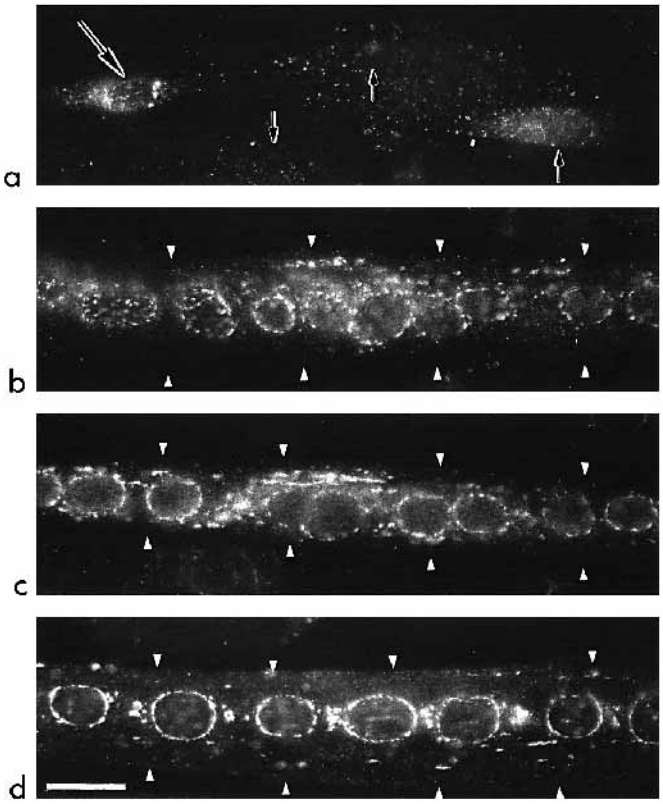

Fig. 1. Immunofluorescence localization of GLUT4 in C2 myoblasts (a), and in myotubes in fusion medium for 1 (b), 3 (c) and 6 days (d). Cultures were fixed and permeabilized as described in Materials and Methods and stained with the anti-GLUT4 antibody followed by fluorescein-conjugated anti-rabbit. Notice in (a) that most myoblasts show only a faint punctate staining (small arrow). Only occasional myoblasts (large arrow) show a stronger staining. The arrowheads in b-d point to the edge of the cell as seen in phase contrast. Most of the staining is restricted to the central core of the myotubes. Bar, 20 μm.

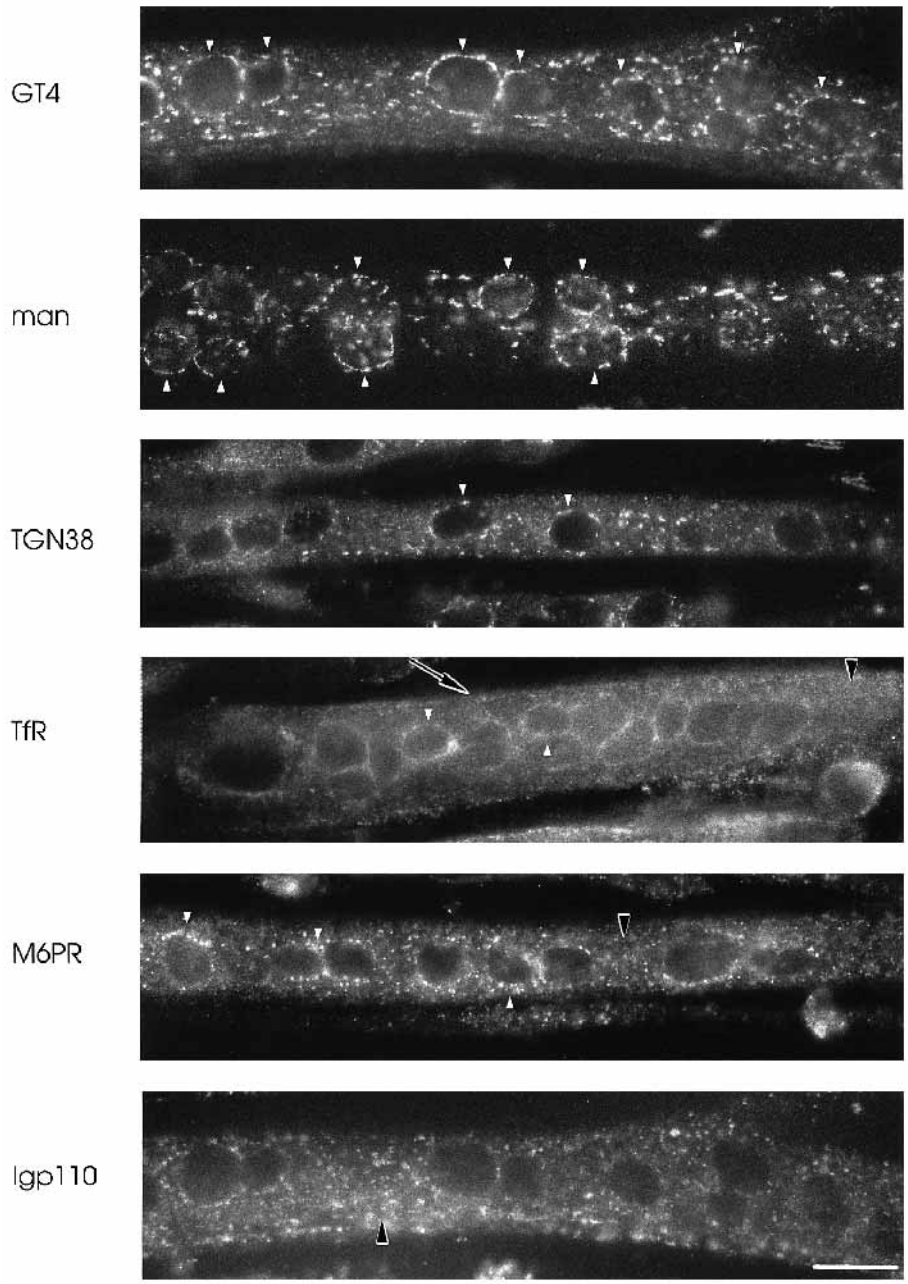

Fig. 2. Comparison of GLUT4 staining to that of markers of various subcellular compartments in C2 myotubes. Cultures of C2 myotubes (3 days in fusion medium) were stained with antibodies to GLUT4, to α mannosidase II (man) as marker of the Golgi complex, to TGN38 as marker of the *trans*-Golgi network, to the transferrin receptor (TfR) as marker of the early endosomes, to the mannose-6-phosphate receptor (M6PR) as marker of the late endosomes and to lgp110 as marker of the lysosomes, followed by the appropriate fluorescein- or rhodamine-conjugated secondary antibody. Notice that most markers are concentrated in the perinuclear area (white arrowheads) and that only TfR gives a plasma membrane staining (arrow). GLUT4 and lgp110 staining were performed simultaneously and the same myotube is photographed under fluorescein (GT4) and rhodamine (lgp110) optics. Large arrowheads point to areas of strong staining in non-perinuclear. Bar, 20 μm.

formation of crossbridges between cisternae of the Golgi complex (Linstedt and Hauri,1993). There is an excellent correlation between the two stainings (Fig. 3), but their simultaneous observation at high magnification (Fig. 4) reveals that they do not totally overlap.

Immunogold electron microscopy (EM) localization (Fig. 5) shows that most grains indicating the presence of GLUT4 are associated with Golgi complexes. The grains are concentrated on one side of the Golgi complex which, based on the distal location in relation to the nuclear membrane (Fig. 5b), must be the *trans* side (Tassin et al., 1985b). They are present in the last cisterna(e) of the Golgi stacks (arrowheads) and in vesicles beyond the cisternae. Such a pattern suggests that GLUT4 is concentrated in the TGN, which is generally defined as including the *trans*-most cisternae of the Golgi complex, and vesicles originating from these cisternae (Mellman and Simons, 1992; Ladinsky et al., 1994; Clermont et al., 1995). GLUT4 grains are also observed scattered in the cytoplasm, but their concentration there is close to background.

When C2 myotubes are stained for GLUT4 after 4 hours of treatment with 20 μg/ml of the protein synthesis inhibitor cycloheximide, there is no evident decrease in perinuclear immunofluorescent staining intensity compared to untreated cultures (not shown). Thus, localization to the Golgi complex is unlikely to result from post-translational transit of GLUT4, which is N-glycosylated (Mitsumoto and Klip, 1992).

GLUT4 is stored in a specific, distinct compartment

To confirm the TGN as the GLUT4 storage compartment, we decided to take advantage of the effects of the fungal metabo-

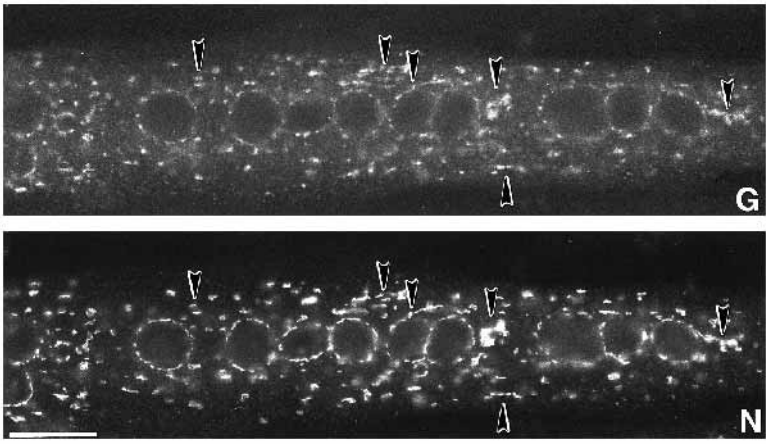

Fig. 3. Comparison between GLUT4 (G) and giantin (N) staining of C2 myotubes. Cultures were double-stained with rabbit anti-GLUT4 and mouse anti-giantin, followed by biotinylated anti-rabbit and Texas red-conjugated avidin, and fluorescein-conjugated anti-mouse. The same myotube is photographed with Texas red (G) and fluorescein (N) filters. At this level of resolution, there is a very good correspondence between the two stainings, as emphasized by arrowheads. Bar, 20 μm.

lite BfA (reviewed by Klausner et al., 1992). Although the effects of BfA vary between cell lines, in most it affects the Golgi complex, the endosomes and the TGN differentially (see Discussion). If this is the case in C2 myotubes as well, BfA should provide some additional information regarding the GLUT4 storage compartment. C2 myotubes were treated for 30 minutes with 10 μM BfA, and stained with the panel of antibodies described previously. All staining patterns (Fig. 6), including that of GLUT4, are dramatically altered, except that for the lysosomal marker lgp110. Mannosidase staining, on the nuclear membrane and in a diffuse pattern throughout the cytoplasm, resembles the pattern of ER markers in myotubes, as expected for markers of the medial Golgi (Doms et al., 1989; Lippincott-Schwartz et al., 1989). Giantin staining is similar to mannosidase (not shown). TGN38, in contrast, presents a strong punctate pattern, also observed with another protein of the TGN, furin (Bosshart et al., 1994; not shown). But the GLUT4 pattern, unexpectedly, is quite distinct from that of mannosidase, giantin, and TGN38; it consists of dots and fine threads that actually resemble TfR and M6PR stainings (see arrowheads). The same pattern was observed after shorter (15 minutes) or longer (2 hours) BfA treatments (not shown).

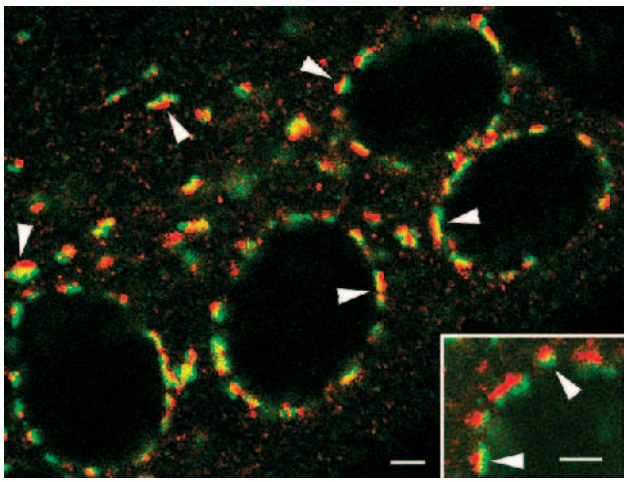

Fig. 4. Myotubes double-stained for GLUT4 and giantin as described in the legend to Fig. 3, but observed in the confocal microscope at high magnification. Although some of the staining appears yellow, indicating superposition of giantin and GLUT4, the colocalization is not complete. Many separate elements show layers of green, yellow and red (arrowheads). The insert shows part of the staining around a single nucleus. The GLUT4 staining (red) always overlaps partially with the giantin staining (green) and extends further away (in the *trans* direction) from the nucleus. Bars, 2.5 μm.

Thus, if GLUT4 is stored in the TGN, it must be in a subcompartment that does not contain TGN38. Alternately, it may be in a membrane compartment geographically close to the TGN.

A connection between GLUT4 and TfR compartments is revealed by BfA

In order to clarify the relationship between GLUT4 and TfR storage vesicles in these myotubes, the cultures were double-stained for the two markers. In cultures without BfA, TfR and GLUT4 immunofluorescent stainings appear distinct (Fig. 7, top two panels), as suggested previously (Fig. 2). In BfA-treated cultures (Fig. 7, bottom two panels), TfR staining shows striking anastomosing tubules, generally aligned with the axis of the myotubes (see also Fig. 5). The GLUT4 pattern appears similar, though the tubules are generally shorter and often fuzzier. The longer of the GLUT4 tubules appear co-localized with the TfR staining (arrowheads). Colocalization is more difficult to assess for the shorter tubules. As a control, BfA-treated cultures were double-stained for TfR and M6PR (Fig. 8). In this case, extensive colocalization was observed after BfA treatment.

C2 myotubes double-stained for GLUT4 and TfR were then observed by electron microscopy. GLUT4 was detected with immunogold, and TfR with immunoperoxidase. In untreated myotubes (Fig. 9a-c), GLUT4 and TfR are often observed close to one another, but in separate membrane systems: TfR in large multivesicular bodies, and GLUT4 in tubulo-vesicular structures that are often next to recognizable Golgi stacks. TfR, as expected, is also found along the plasma membrane, in coated pits and in coated and uncoated vesicles (Fig. 9a). In this part of the cell, GLUT4 staining is found as single grains, at a density that is similar to that of the background, or in small aggregates of 2-4 grains, which do not colocalize with immunoperoxidase (not shown).

In BfA-treated cultures (Fig. 10), the patterns of both GLUT4 and TfR change strikingly. No large concentration of GLUT4 silver grains is observed near the nuclei or anywhere, and the grains are more scattered. Often they can be seen aligned on thin tubules of diameter ~70 nm (Fig. 10a-d). TfR

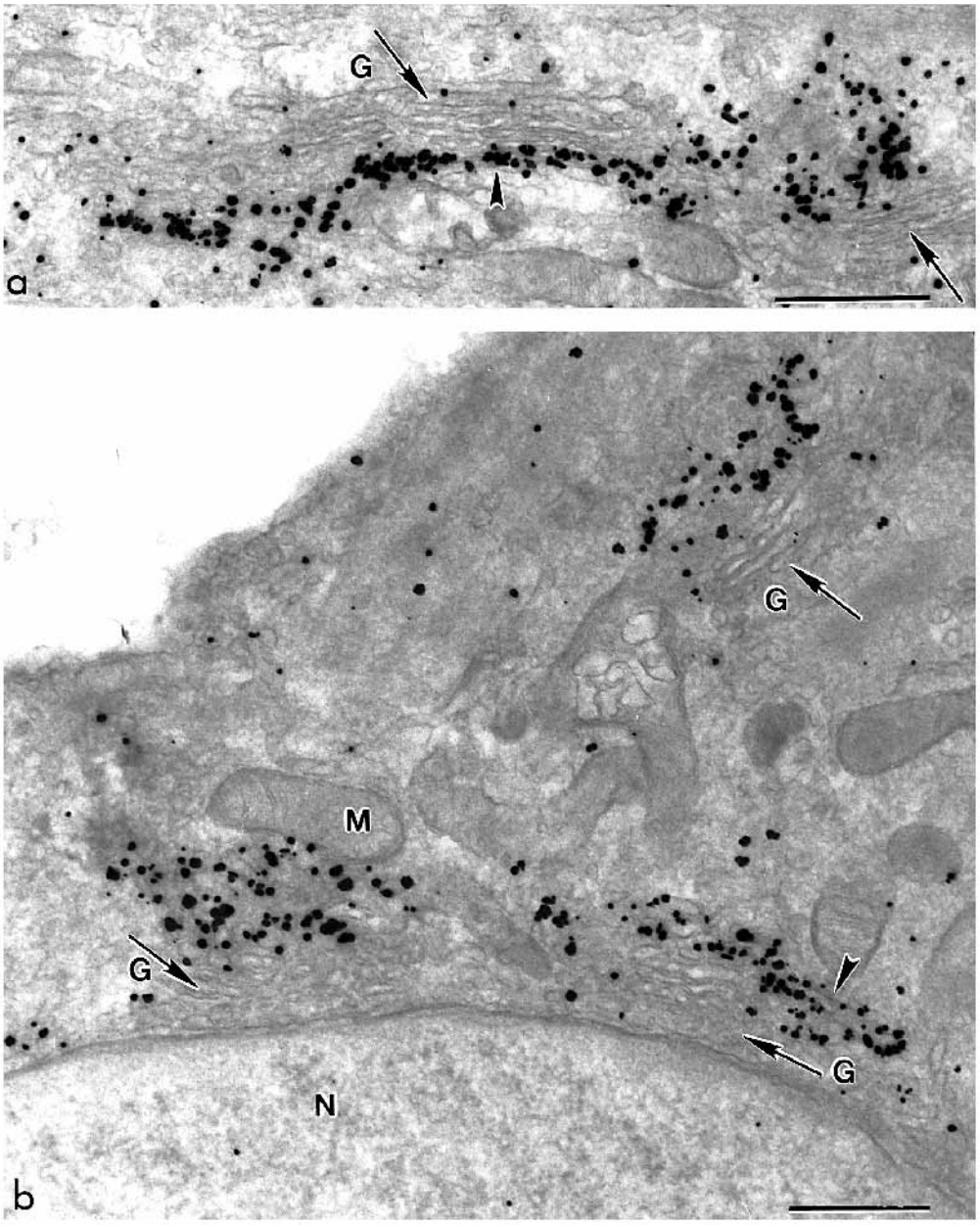

Fig. 5. Electron micrograph showing immunogold staining for GLUT4 (see Materials and Methods). (a) In an area of the cell that is not near a nucleus, two ribbons of Golgi cisternae (arrows) appear twisted. The GLUT4 grains (arrowheads) are clearly on one side of the ribbon, and follow the course of the cisternae on the left, but appear to be mostly in vesicles on the right. (b) Three sets of Golgi cisternae, two of which are close to a nucleus (N), show heavy labeling of the *trans*-most cisternae (arrowhead) or of vesicles further beyond. Bars, 500 nm.

is found on the tubules as well, and on varicosities along the tubules. Following BfA treatment, the previously separate TfR and GLUT4 compartments now appear to have fused to form a network of tubules and still recognizable vesicles (the varicosities). In this network, diffusion of TfR and GLUT4 may not be completely free since they are not entirely superimposed (see Fig. 10c, for example).

GLUT4 vesicles are associated with microtubules

To determine if microtubules are involved in the effects of BfA on GLUT4 redistribution, cultures were treated with 1 μg/ml nocodazole for 2 hours. Staining with an antibody against α-tubulin confirmed that microtubules had disappeared, except for some very short fragments radiating from the nuclear membrane (not shown). The cultures appeared healthy. In nocodazole-treated cultures stained for GLUT4 (Fig. 11), the perinuclear staining can still be observed but it appears fragmented. Similarly, large aggregates between nuclei appear mostly fragmented and dispersed. When BfA is added to nocodazole-treated cultures (Fig. 11), large flat aggregates form, but no tubules can be found. Nocodazole also prevents the tubularization of TfR staining (not shown). Thus, GLUT4-containing vesicles must be associated with microtubules, along which the tubules observed after BfA treatment extend.

DISCUSSION

In this work, we have addressed two often debated questions that are key issues in understanding glucose transport: the nature and the specificity of the GLUT4 storage site. Because of the importance of muscle for insulin-stimulated glucose

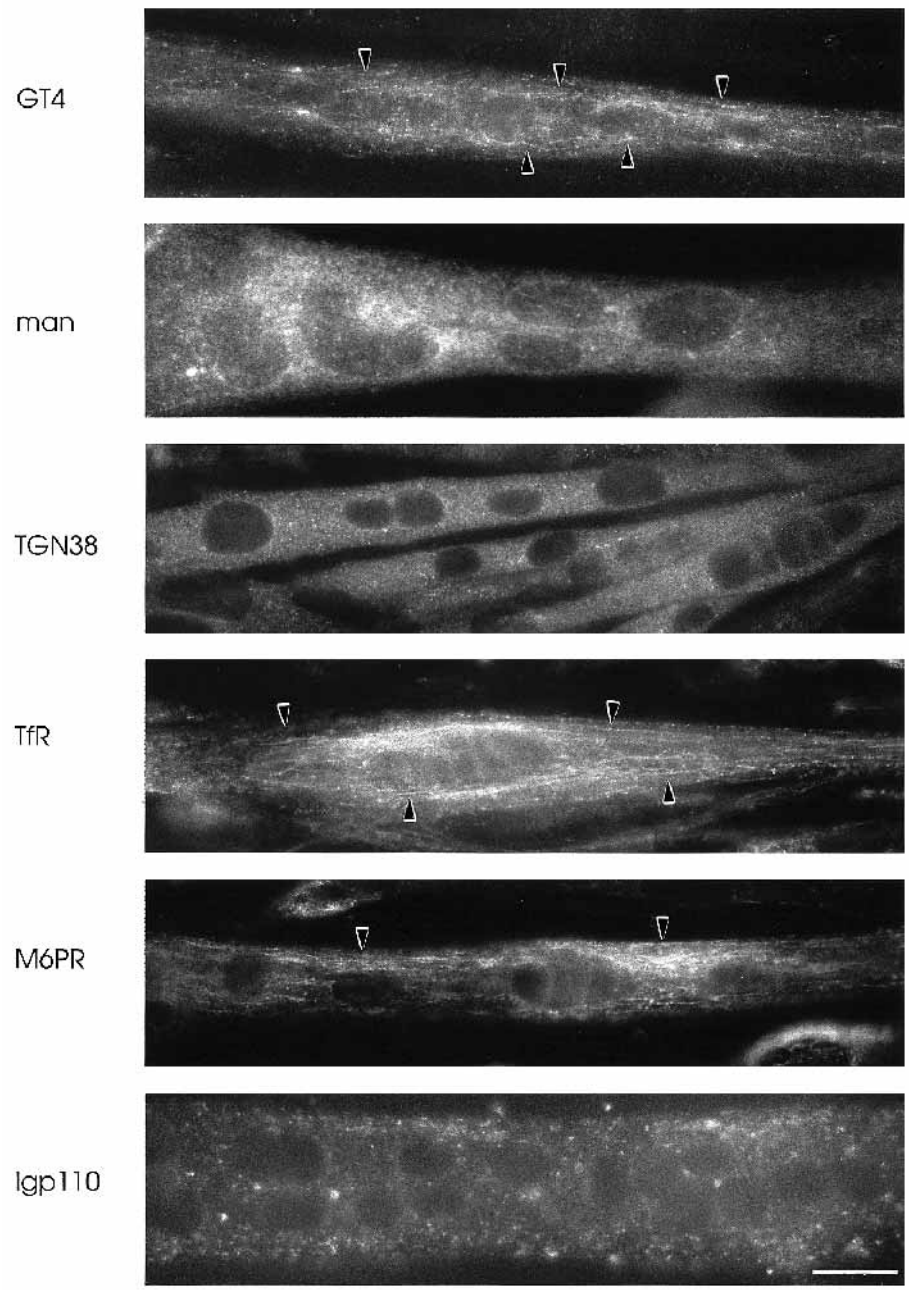

Fig. 6. Localization of markers of various subcellular compartments in BfA-treated C2 myotubes. The staining was identical to that described in Fig. 2, but cultures had been treated for 30 minutes with 10 μg/ml brefeldin A before fixation. All panels show different myotubes. Arrowheads emphasize thread-like staining of GLUT4 and of endosomal markers. Bar, 20 μm.

disposal (De Fronzo, 1992), we have choosen to examine GLUT4 in cultured myotubes, which express GLUT4 as part of their normal development, and respond to insulin stimulation (Ramlal et al., 1988; Galante et al., 1995). We conclude that GLUT4, in unstimulated muscle cells, is segregated from the transferrin receptor and from several available markers of the endosomal-lysosomal pathway and stored in a specific tubulo-vesicular compartment structurally resembling the TGN. But although GLUT4 and TfR are not colocalized, we find that there is a functional link between the two compartments, most likely via microtubules.

When the possible colocalization of GLUT4 and markers of early and late endosomes, Golgi complex, TGN and lysosomes is assessed by immunofluorescence (Fig. 2), the GLUT4 pattern differs from that of TfR but strikingly resembles that of Golgi complex markers. Double staining of myotubes for GLUT4 and giantin (Fig. 3) confirms a close association between GLUT4 and the Golgi complex, which we also observe in rat muscle fibers in vivo (T. Ploug et al., unpublished). Immunogold EM localization of GLUT4 (Fig. 5) shows staining of the *trans*-most Golgi cisterna(e) and of vesicles beyond, which together structurally define the TGN (Mellman and Simons, 1992; Ladinsky et al., 1994; Clermont et al., 1995). TGN localization of GLUT4 would explain that GLUT4 and giantin only partially overlap at high resolution (Fig. 4), since giantin is believed to be in the cisternal part of the Golgi complex (Linstedt and Hauri, 1993). The ability of confocal microscopy to resolve different compartments of the Golgi complex has been established by others (Antony et al., 1992; Nilsson et al., 1993).

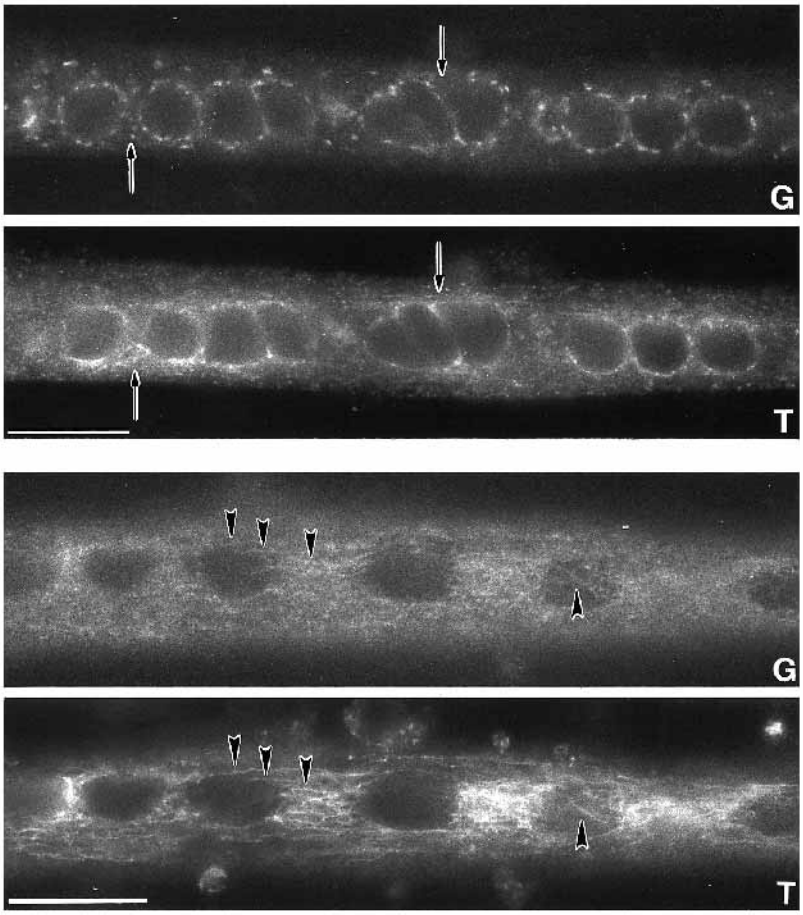

Fig. 7. Simultaneous localization of TfR (T) and GLUT4 (G) in C2 myotubes, without (top two panels) or with BfA treatment (bottom two panels). Cultures similar to those described in Figs 5 and 6 were double stained with rabbit anti-GLUT4 and rat anti-TfR, followed by biotinylated anti-rabbit IgG and fluorescein-conjugated anti-rat IgG and by Texas red-conjugated avidin. Arrows point to features that stand out in one staining only, whereas arrowheads point to features that can be recognized in both. Bars, 20 μm.

In order to further explore the connection between GLUT4 and the TGN, we decided to take advantage of the effects of the fungal metabolite brefeldin A. Although the best known effects of BfA are its redistribution of Golgi complex markers to the ER (Doms et al., 1989; Lippincott-Schwartz et al., 1989), BfA has been observed to affect TGN, endosomes and lysosomes as well (Lippincott-Schwartz et al., 1991; Wood et al., 1991), fusing them into long tubules. We reasoned that the differential effect of BfA on the different compartments of the Golgi complex should provide additional evidence for localization of GLUT4 to one of them.

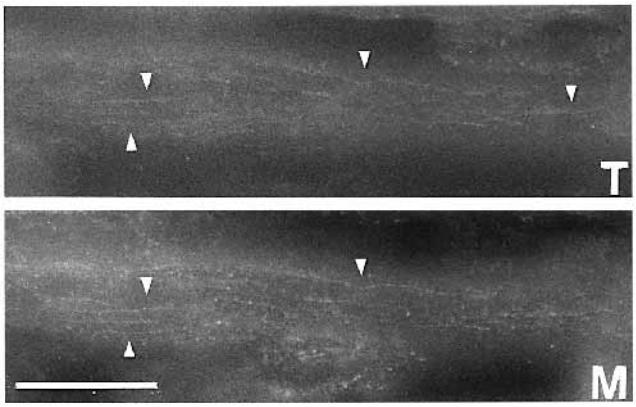

Fig. 8. Simultaneous localization of TfR (T) and M6PR (M) in C2 myotubes treated with BfA. Cultures similar to those described in Fig. 7 were double-stained with rat anti-TfR and rabbit anti-M6PR, followed by fluorescein-conjugated anti-rat IgG (for TfR) and biotinylated anti-rabbit IgG, followed by Texas red avidin (for M6PR). Arrowheads point to features that can be recognized in both panels. Bar, 20 μm.

The effects of BfA are not universal, however, since some cells show resistance or a different reaction to the drug (Sandvig et al., 1991). There has not been any previous morphological study of the effects of BfA on organelles in muscle cells. It was thus necessary to first establish how C2 myotubes respond to BfA treatment. Except for lgp110, all markers examined (Fig. 6), including GLUT4, were dramatically redistributed in BfA-treated myotubes. Resistance of lysosomes to BfA has been noticed in some other cells (Prydz et al., 1992). For several markers, the changes were as expected from observations in other cells: redistribution of mannosidase to the ER, and tubularization of TfR and of M6PR endosomes. However, some features can be attributed to the unique geometry of multinucleated myotubes: even after long treatments with BfA, the staining for TfR or M6PR does not 'collapse' to the centrosome, as observed in mononucleated cells (Lippincott-Schwartz et al., 1991), most likely because of the inactivation of the centrosomes during myogenesis (Tassin et al., 1985a,b; Ralston, 1993). In contrast, the dispersion of TGN38 by BfA differs from the tubularization observed in fibroblast-like cells, but is similar to what is observed in neocortical neurons (Lowenstein et al., 1994). As to GLUT4, it is induced by BfA to form tubular structures resembling those of TfR but totally

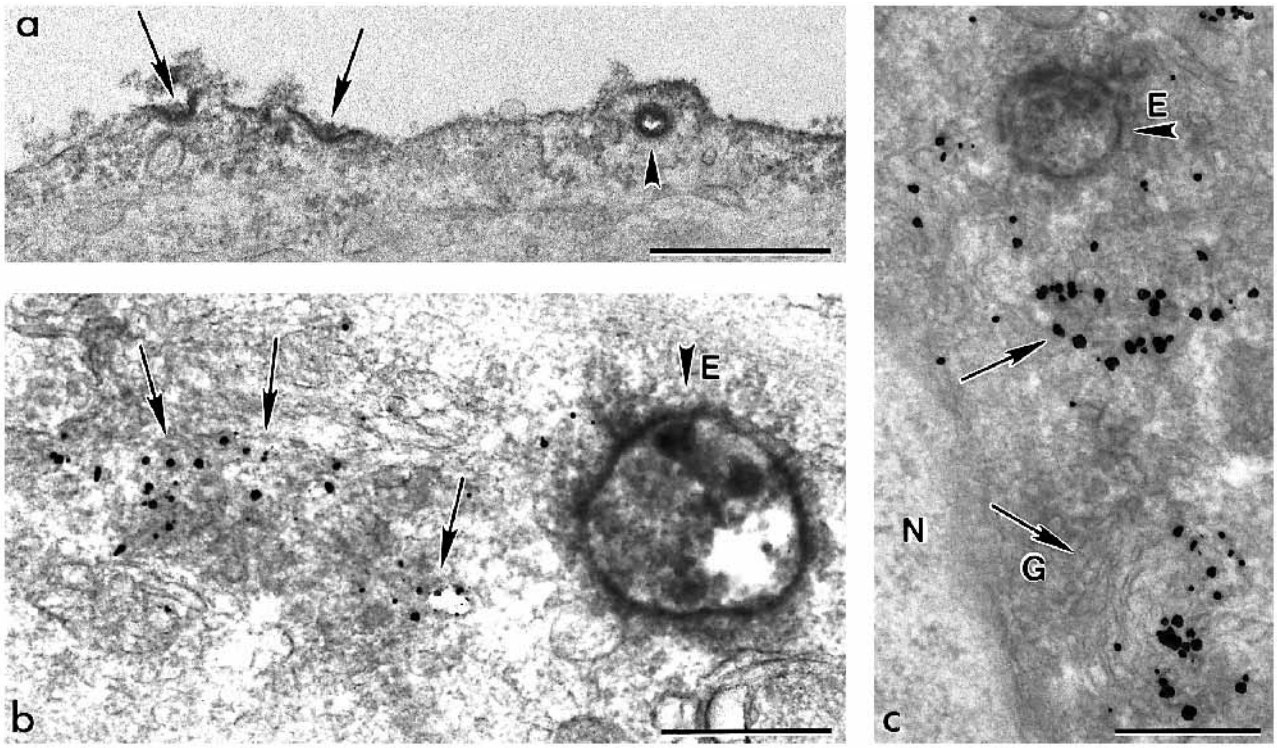

Fig. 9. Electron micrographs showing TfR and GLUT4 staining in C2 myotubes. (a) Staining for TfR only, observed in a thin (~70 nm) section. Arrows point to coated pits and the arrowhead to what appears to be a vesicle near a labeled section of plasma membrane. (b) A thicker section (~200 nm), with immunogold grains indicating GLUT4 (arrows) in tubulovesicular structures very close to a large multivesicular endosome (E), labeled with immunoperoxidase (arrowhead), indicating TfR. (c) Another area, again showing a TfR-containing endosome (E, arrowhead), close to but distinct from immunogold labeling of GLUT4 (arrows), in a Golgi area (G) and in non-characteristic tubulo-vesicular elements close to a nucleus (N). Bars, 500 nm.

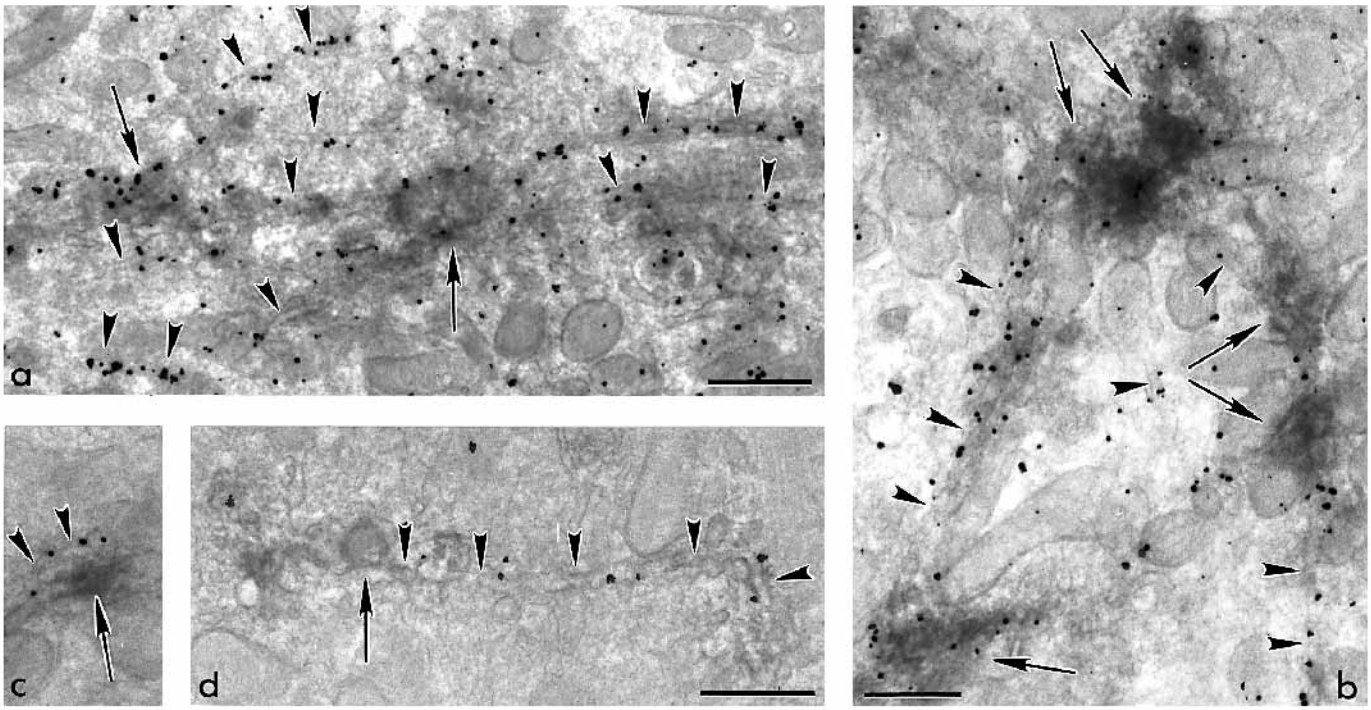

Fig. 10. Electron micrographs showing TfR and GLUT4 staining in C2 myotubes treated with BfA. (a-c) Thick (~200 nm) sections; (d) thin (~70 nm) section. Immunoperoxidase staining (arrows) and immunogold (arrowheads) are mostly on a network of tubules and varicosities. Bars, 500 nm.

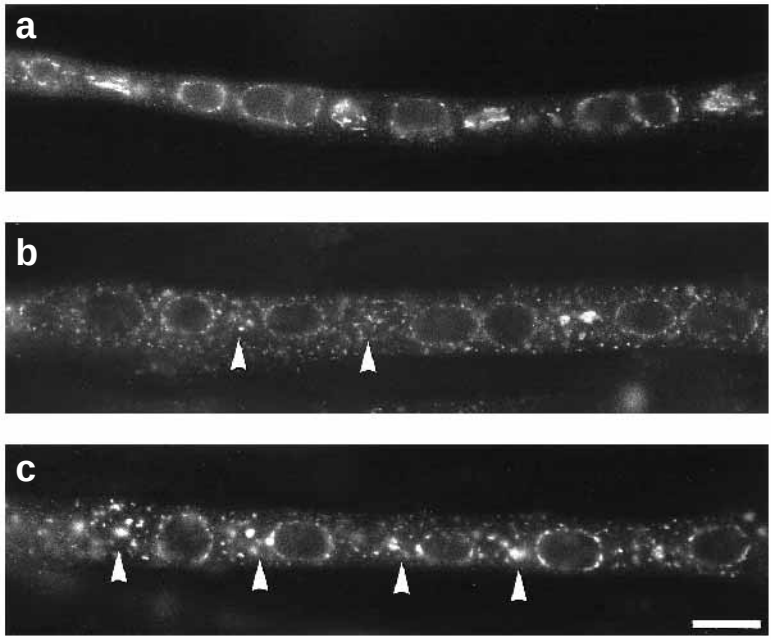

Fig. 11. Microtubule depolymerization blocks BfA effects on GLUT4. C2 myotubes were treated with 1 μg/ml nocodazole (N) for 2 hours and then stained for GLUT4. In some cultures, BfA (10 μg/ml) was added for the last 30 minutes. (a) Control culture; (b) culture treated with nocodazole alone; (c) culture treated with nocodazole and BfA. Arrowheads point to fragmented pattern in nocodazole-treated cultures and to large flat aggregates in BfA and nocodazole-treated cultures. Bar, 20 μm.

distinct from the markers of the Golgi proper or the TGN. This result was surprising in view of the strong EM evidence that GLUT4 is associated with the Golgi complex but it is consistent with a report (Martin et al., 1994) that there is very little, if any, colocalization of GLUT4 and TGN38 in subcellular fractions of 3T3-L1 adipocytes.

The simplest interpretation of our results is that GLUT4 is not in the TGN, since its pattern diverges from that of TGN38, but is geographically close to it. This interpretation is difficult to sustain in view of the EM pattern of GLUT4. However, the TGN is a complex structure, whose geometry and relative importance vary between cells (Clermont et al., 1995). A second interpretation is that GLUT4 is in a TGN subcompartment that does not contain TGN38. In favor of this interpretation is the observation that p200, another marker of the TGN, colocalizes only partially with TGN38 (Narula and Stow, 1995). In NRK cells, BfA causes tubularization of the TGN38 pattern (Lippincott-Schwartz et al., 1991) but dispersion of p200 (Narula et al., 1992). p200 itself may not be involved in transport of proteins to the cell surface (Ikonen et al., 1996), but there may still be other unknown markers of TGN subcompartments. Finally, we cannot exclude at this point the possibility that the vesicles labeled with TGN38 do not represent, in myotubes, the functional TGN, where sorting of proteins takes place before vesicles bud off to various destinations. TGN38 is downregulated during myogenesis, and is undetectable in muscle fibers in vivo (Jasmin et al., 1995). Therefore, GLUT4 might label the 'real' TGN, and its tubular pattern and connection with the TfR endosomes in BfA-treated cells would be easily explained. Clearly, more work will be required to solve this interesting question, which has implications beyond the immediate goals of the present paper.

Although GLUT4 staining in the Golgi complex region has been reported for brown adipose cells (Slot et al., 1991) and transfected L6 myoblasts (Haney et al., 1995), a close association has not been noticed. In EM sections, labeling is mostly of vesicles and the associated cisternae are not always visible (see Fig. 9, for example); and in light microscopy, the compact shape of the Golgi complex in mononucleated cells makes colocalization difficult to assess. In contrast, the unique architecture of the Golgi complex in myotubes, especially its fragmented appearance, facilitates the interpretation of colocalization experiments such as the giantin-GLUT4 staining (Fig. 3). In addition, the EM pre-embedding staining approach followed here provides a high efficiency of labeling and large sections to observe.

The demonstration that GLUT4 is redistributed following BfA treatment has not been made previously, although several studies have examined whether BfA perturbs glucose transport. BfA inhibited glucose transport in adipocytes in one study (Lachaal et al., 1994), but not in two others (Chakrabarti et al., 1994; Bao et al., 1995) or in L6 myotubes (Hundal et al., 1994). The density of GLUT4-containing fractions did not seem affected by BfA either (Hundal et al., 1994), leading to the interpretation that BfA has no effect on GLUT4 distribution. We show this not to be the case. A partial explanation of these apparently contradictory results is that BfA may change the distribution of a protein without affecting its recycling, as shown for TfR (Klausner et al., 1992).

We observe segregation of GLUT4 and TfR, both by immunofluorescence (Figs 2 and 7) and by immunogold EM (Fig. 9). In the central part of the myotubes, TfR is observed in large multivesicular bodies (Fig. 9b-c), presumably endosomes (van Deurs et al., 1993). GLUT4 EM immunogold staining is on tubulovesicular structures, most of them next to identifiable Golgi complexes. These tubulovesicular structures are often very close to the endosomes, sometimes to the point of appearing connected to them, but the staining for GLUT4 and TfR does not overlap. These results are especially important since it is the first time that GLUT4 and TfR have been observed simultaneously in whole cells at the EM level. Most previous studies of colocalization were based on fractionation studies, and the only immunofluorescence study (Hudson et al., 1992) was on transfected non-insulin-respon-

sive cells. Although fractionation studies have undoubtedly been important in unraveling several aspects of glucose transport, it is not always possible to determine where in the intact cells the fractions originate from. This is especially true when traditional markers are not present in these fractions, as is the case for GLUT4 vesicles, which were recently suggested to be free vesicles (Kandror et al., 1995). The present work suggests, in contrast, that these vesicles may originate from the TGN. The distinction is important for our understanding of GLUT4 trafficking.

Finally, the demonstration, by BfA, of an underlying connection between the separate TfR and GLUT4 compartments provides a basis for reconciliation of the apparently contradictory observations of the coordinate translocation of TfR and GLUT4 following insulin stimulation, and of their segregation in different compartments.

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