The T-tubule is a cell-surface target for insulin-regulated recycling of membrane proteins in skeletal muscle

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(1) In this study we have determined the distribution of various membrane proteins involved in insulin-activated glucose transport in T-tubules and in sarcolemma from rat skeletal muscle. Two independent experimental approaches were used to determine the presence of membrane proteins in T-tubules: (i) the purification of T-tubules free from sarcolemmal membranes by lectin agglutination, and (ii) T-tubule vesicle immunoadsorption. These methods confirmed that T-tubules from rat skeletal muscle were enriched with dihydropyridine receptors and tt28 protein and did not contain the sarcolemmal markers dystrophin or β_1-integrin. Both types of experiments revealed an abundant content of GLUT4 glucose carriers, insulin receptors and SCAMPs (secretory carrier membrane proteins) in T-tubule membranes. (2) Acute administration *in vivo* of insulin caused an increased abundance of GLUT4 in T-tubules and sarcolemma. On the contrary, insulin led to a 50% reduction in insulin receptors present in T-tubules and in sarcolemma, demonstrating that insulin-induced insulin receptor internalization affects T-tubules in the muscle fibre. The alteration in the content of GLUT4 and insulin receptors in T-tubules was a consequence of insulin-induced redistribution of these proteins. SCAMPs also redistributed in muscle membranes in response to insulin. They were recruited by insulin from intracellular high-density fractions to intracellular lighter-density fractions and to the cell surface, showing a pattern of insulin-induced cellular redistribution distinct from those of GLUT4 and the insulin receptor. (3) In conclusion, the T-tubule is a cell-surface target for membrane proteins involved in recycling such as SCAMPs or for membrane proteins that acutely redistribute in response to insulin such as GLUT4 or insulin receptors.

INTRODUCTION

In adult skeletal muscle, the signal for excitation–contraction coupling is transferred across the triad junction from transverse tubules (T-tubules) to the terminal cisternae of the sarcoplasmic reticulum to trigger Ca^{2+} release and muscle contraction. Recent evidence indicates that T-tubules are sites for the initiation of certain signalling pathways, which might be connected with the role of T-tubules in the transmission of membrane depolarization. Thus, T-tubules contain enzymes and other components important for the phosphoinositide pathway, including G-proteins [1–5]. In this regard, phospholipase C activity, and phosphatidylinositol 4,5-bisphosphate and inositol 1,4,5-trisphosphate are detected in T-tubules [3–5]. Protein kinase C is also found in T-tubules and its abundance in this cell-surface site is greater than in the sarcolemma [6,7].

The T-tubules could also have an important role in the uptake of energy sources. In this regard, GLUT4 glucose transporters have been detected in T-tubules from skeletal muscle and insulin has been shown to increase GLUT4 abundance in T-tubules [8–11], although there is some controversy on this aspect [12,13]. The existence of glucose uptake through T-tubules would fit with the fact that glycolytic enzymes show a heterogeneous distribution in the muscle fibre and they are preferentially found in a cytosolic compartment surrounding the I band, i.e. close to T-tubules [14–16]. Furthermore, insulin increases the binding of hexokinase to mitochondria in muscle [17,18], which are found peripherally close to the sarcolemma and packed in between the myofibrils, preferentially in a transverse plane between the triadic system and the Z disk, i.e. close to the T-tubules [19]. In consequence, the arrival of glucose through the T-tubule would permit the direct channelling of glucose to glycolytic enzymes located close to T-tubules.

Based on the fact that skeletal muscle is quantitatively the most important tissue of insulin-stimulated glucose disposal [20], we have studied the distribution of GLUT4 and insulin receptors in skeletal-muscle membranes obtained under basal and insulin-stimulated conditions. In order to provide information on the fine topology of GLUT4 glucose transporters and insulin receptors, we made use of a protocol set up in our laboratory for the extraction of highly purified fractions of sarcolemmal and T-tubule origin from a single preparation of rat skeletal muscle [21]. Subcellular membrane fractionation and vesicle immunoadsorption were used to determine the distribution of glucose carriers and insulin receptors in skeletal muscle under basal and insulin-stimulated conditions. Based on the recent identification of SCAMPs (secretory carrier membrane proteins) as markers within the general cell-surface recycling systems [22,23], we have also evaluated the distribution of these proteins in different membrane fractions from skeletal muscle.

MATERIALS AND METHODS

Materials

^{125}I-labelled Protein A and ^{125}I-labelled sheep anti-(mouse IgG) antibody were purchased from ICN. ECL reagents were from Amersham. Immobilon polyvinylidene difluoride (PVDF) membranes were obtained from Millipore. All electrophoresis reagents and molecular mass markers were obtained from Bio-

Abbreviations used: T-tubule, transverse tubule; SCAMP, secretory carrier membrane protein; PVDF, polyvinylidene difluoride; WGA, wheat germ agglutinin.

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Rad. γ-Globulin, wheat germ agglutinin (WGA), goat anti-(mouse IgG) and goat anti-(mouse IgM) antibodies coupled to agarose and most commonly used chemicals were from Sigma Chemical Co., St. Louis, MO, U.S.A.

Antibodies

Both monoclonal (1F8) and polyclonal (OSCRX) antibodies specific for GLUT4 were used in these studies. Monoclonal antibody 1F8 [24] was used for immunoisolation assays. Anti-GLUT4 (OSCRX) antibody from rabbit was produced after immunization with a peptide corresponding to the final 15 amino acids of the C-terminus [25]. A polyclonal antibody against the C-terminus of the rat insulin receptor (residues 1341–1357) was kindly given by Dr. Willy Stalmans (Katholieke Universiteit, Leuven, Belgium) [26]. Monoclonal antibody 3F8 was used to immunodetect GTV3/SCAMP proteins [27]. A rabbit polyclonal antibody against rat β_1-integrin was kindly given by Dr. Carles Enrich (University of Barcelona) [28]. A rabbit polyclonal antibody against the α_2 component of Ca^{2+} channels (dihydropyridine receptors) [29] was obtained from Dr. Michel Lazdunski (Centre de Biochimie, Centre National de la Recherche Scientifique, Sophia Antipolis, France). Monoclonal antibody NCL-DYS 1 against the mid rod of dystrophin was obtained from Novocastra. Monoclonal antibody A-52 against Ca^{2+}-ATPase [30] was kindly donated by Dr. David H. MacLennan (University of Toronto, Canada). Monoclonal antibody TT-2, which recognizes protein tt28 (specific to T-tubules [31]), was also used in these studies. Monoclonal antibody SY38 against synaptophysin was obtained from Boehringer Mannheim.

Animals and tissue sampling

Male Wistar rats weighing between 250 and 300 g from our own colony were fed with Purina Laboratory chow *ad libitum* and housed in animal quarters maintained at 22 °C with a 12-h light, 12-h dark cycle. After an overnight fast, rats were anaesthetized with sodium pentobarbital and some were injected with insulin (intravenously, 10 units/kg body weight) and D-glucose (intraperitonealy, 1 g/kg body weight) 30 min before tissue removal. White portions of gastrocnemius and quadriceps muscles were then rapidly excised and immediately processed. Muscle enriched in white fibres was used because of its greater content of T-tubule membranes [32].

Isolation of rat skeletal muscle membranes

The different cell-surface and intracellular membrane fractions were isolated as reported [21]. Approximately 12 g of rat skeletal muscle was excised, weighed, minced and initially homogenized with a Polytron at low speed (setting 4, 2 × 5 s) in buffer A (20 mM Tris/HCl, 0.25 M sucrose, 1 mM EDTA, 1 μM pepstatin, 1 μM leupeptin, pH 7.4; 1 g of tissue/4 ml of buffer A). The homogenate was centrifuged for 20 min at 12000 ***g***. The supernatant was collected and kept on ice. The pellet was resuspended in buffer A and centrifuged again for 20 min at 12000 ***g***. The two supernatants were pooled and were referred to as the F1 fraction. The pellet was resuspended in buffer A and subjected to a high-speed homogenization (Polytron at setting 6, 2 × 30 s). The homogenate was centrifuged for 20 min at 12000 ***g*** and the supernatant was collected and referred to as F2. F1 and F2 fractions were incubated with 0.6 M KCl for 1 h at 4 °C and then pelleted for 1 h at 150000 ***g***. The pellets from KCl-washed F1 and F2 fractions were then subjected to calcium-loading in order to increase the density of sarcoplasmic reticulum vesicles [33]. To this end, pellets were resuspended in buffer B (50 mM potassium phosphate, 5 mM $MgCl_2$, 150 mM KCl, pH 7.5) at a protein concentration of 2 mg/ml. Calcium loading was initiated by addition of 0.3 mM $CaCl_2$ and 2 mM ATP. After incubation for 20 min at room temperature, F1 and F2 fractions were centrifuged for 60 min at 150000 ***g***. F1 and F2 pellets were resuspended in buffer C (20 mM Tris/HCl, 50 mM sodium pyrophosphate, 0.3 M KCl, 0.25 M sucrose, pH 7.2) and layered on top of a discontinuous density gradient consisting of 3 ml of 35 %, 2 ml of 29 %, 2 ml of 26 % and 2 ml of 23 % (w/v) sucrose. After centrifugation for 12 h at 77000 ***g***, four protein fractions were separated from F1 and F2 fractions: fraction 23 on top of the 23 % layer; fraction 26 from the interphase 23–26 %; fraction 29 from the interphase 26–29 %; fraction 35 from the interphase 29–35 %. In some experiments, the pellet resulting from this centrifugation was also collected (pellet-F1 and pellet-F2). All the fractions were collected, diluted with 20 mM Tris/HCl, pH 7.4, and centrifuged for 60 min at 150000 ***g***. Pellets were resuspended in 30 mM Hepes, 0.25 M sucrose, pH 7.4. Protein concentrations were determined by the method of Bradford [34] using γ-globulin as a standard.

Isolation of purified sarcolemma and T-tubules by wheat germ agglutination

To purify the surface membrane preparations (23F1 and 23F2), vesicles were treated with WGA [35,36]. Surface membranes were resuspended at a protein concentration of 1 mg/ml in buffer D (50 mM sodium phosphate, 160 mM NaCl, pH 7.4) and mixed with an equal volume of 1 mg/ml wheat germ lectin in buffer D. The total volume of this mixture was 600 μl and after 10 min incubation in ice, the solution was pelleted in a microfuge for 1.5 min at 15000 ***g*** (13000 rev./min). The lectin-agglutinated vesicles (W^+ fractions) were resuspended in buffer E (20 mM Tris/HCl, 0.250 M sucrose, pH 7.4) and centrifuged as described above. This procedure was repeated and the resuspended pellets were then de-agglutinated by incubation for 20 min at 0 °C in 500 μl of 0.3 M *N*-acetyl-D-glucosamine in buffer E. The de-agglutinated suspension was centrifuged in a microfuge for 1.5 min at 15000 ***g*** and the supernatant was pelleted at 150000 ***g*** for 60 min (in a TLS-55 Beckman rotor). The pellet was resuspended in buffer E and frozen in liquid nitrogen. The non-agglutinated vesicles (W^- fractions) were centrifuged for 60 min at 150000 ***g***. The pellet was resuspended in buffer E and stored frozen.

Protocols of vesicle immunoisolation

Antibodies 1F8 (5–7 μg) and TT-2 (1–3 μl) were incubated overnight at 4 °C with goat anti-(mouse IgG)- or goat anti-(mouse IgM)-coupled agarose (75 μl bead volume). Beads were collected by a 6 s spin in a microfuge and washed in PBS. Intact membrane preparations (15–25 μg of protein) were incubated with 1F8- or TT-2-Ig–agarose overnight at 4 °C in the absence of detergents (0.1 % BSA, 1 mM EDTA in PBS; final volume 200 μl). The agarose beads and vesicles bound to them were collected by a 6 s spin in a microfuge. The vesicles that were bound to the immobilized antibody were washed in PBS. Then the beads were incubated in electrophoresis sample buffer, incubated for 5 min at 95 °C, cooled and microcentrifuged. The supernatant fraction from the vesicle immunoadsorption assay and the immunoadsorbed extract were subjected to SDS/PAGE.

Electrophoresis and immunoblot analysis

SDS/PAGE was performed on membrane proteins following the method of Laemmli [37]. For immunodetection of most proteins,

non-reducing 8.5, 10 or 12% polyacrylamide gels were used. For immunodetection of the β-subunit of the insulin receptors and dystrophin, samples were reduced with 100 mM dithiothreitol and run in 6% gels. Proteins were transferred to Immobilon as previously reported [38] in buffer consisting of 20% methanol, 200 mM glycine, 25 mM Tris, pH 8.3. Following transfer, the filters were blocked with 5% non-fat dry milk/0.02% sodium azide in PBS for 1 h at 37 °C and were incubated with antibodies in 1% non-fat dry milk/0.02% sodium azide in PBS. Transfer was confirmed by Coomassie Blue staining of the gel after the electroblot. Detection of the immune complex with the rabbit polyclonal antibodies was accomplished using ^{125}I-Protein A for 4 h at room temperature. Detection of the immune complex with monoclonal antibodies was performed using ^{125}I-labelled sheep anti-mouse antibody. Antibody 3F8 was detected using horseradish peroxidase linked to goat anti-(mouse IgM) secondary antibody and visualized using an enhanced chemiluminescence system (ECL, Amersham Corp.). The autoradiograms were quantified using scanning densitometry. Immunoblots were performed under conditions in which autoradiographic detection was in the linear response range.

RESULTS

T-tubules contain GLUT4 glucose carriers, insulin receptors and SCAMPs

The distribution in the muscle fibre of membrane proteins involved in insulin action was studied by using a protocol of subcellular fractionation that involves the preparation of two crude membrane fractions by sequential homogenization at low speed (F1) and high speed (F2) starting from a single preparation of muscle tissue [21] (Figure 1). Upon centrifugation in sucrose gradients, membrane collected from the top of the 23% sucrose layer (23F1 and 23F2) represents a crude cell-surface fraction that can be further separated into two subdomains of sarcolemma and a T-tubule fraction (Figure 1). The specific proteins selected for study were insulin receptors, GLUT4 glucose carriers and SCAMPs. These proteins showed marked differences in subcellular distribution in skeletal muscle. Insulin receptors were mainly detected in cell-surface membrane fractions, similarly to the distribution of sarcolemmal markers (β_1-integrin and dystrophin) or T-tubule markers (dihydropyridine receptors and protein tt28) (Table 1). In contrast, GLUT4 was mainly localized in intracellular membrane fractions. Antibody 3F8 [27] against SCAMPs recognized two bands from membrane preparations of skeletal muscle, showing apparent molecular masses of 39 kDa and 37 kDa. Both SCAMP 39 and SCAMP 37 were also mainly localized in intracellular membranes (Table 1). Both the cell-surface membranes and intracellular membranes obtained after subcellular fractionation of rat skeletal muscle did not contain membranes of peripheral nerve origin, as judged by the absence of synaptophysin (results not shown).

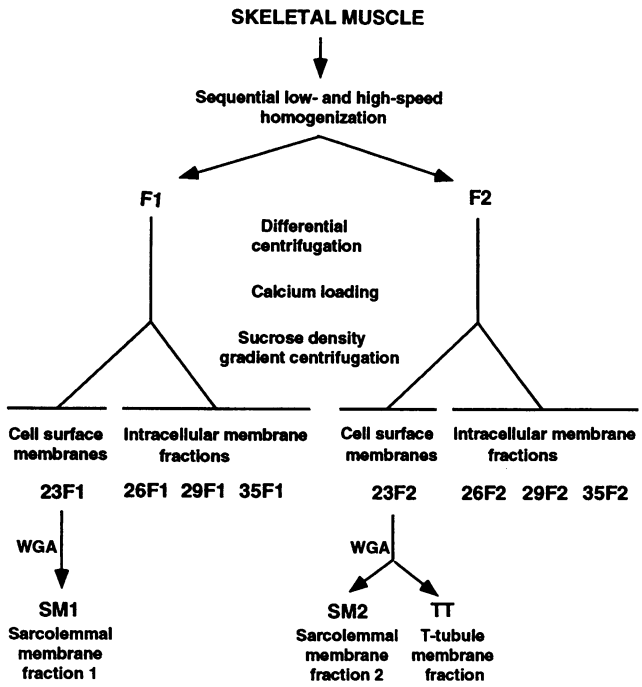

Figure 1 Flow chart of procedure to isolate different membrane fractions from rat skeletal muscle

The detection of membrane proteins in T-tubules was analysed after purification of T-tubule membrane vesicles by using two alternative experimental procedures: (a) purification of T-tubule membranes by wheat germ agglutination, and (b) immunoadsorption of T-tubule membrane vesicles.

We have recently reported the isolation of three distinct cell-surface domains from rat skeletal muscle: sarcolemmal fraction 1 (SM1), sarcolemmal fraction 2 (SM2) and a T-tubule fraction obtained by subcellular fractionation that incorporates a final step of agglutination in WGA [21]. In order to determine whether glucose carriers, insulin receptors and SCAMPs were present in T-tubule membranes, Western blotting of these proteins was performed on fractions SM1, SM2 and T-tubules. In keeping with previous observations [21], fraction SM1 was characterized by a high abundance of β_1-integrin and it was dystrophin-free; fraction SM2 was highly enriched in both β_1-integrin and dystrophin; and the T-tubule fraction was highly enriched in dihydropyridine receptors and was essentially devoid of membranes of sarcolemmal origin (Figure 2a). These different cell-surface membranes did not show contamination with sarcoplasmic reticulum vesicles, as judged by the absence of sarcoplasmic Ca^{2+}-ATPase (results not shown).

The utilization of membranes from sarcolemmal and T-tubule origin showed a pattern of distribution for each membrane protein subjected to study. GLUT4 was present in all cell-surface fractions, i.e. SM1, SM2 and T-tubules, and GLUT4 abundance was significantly greater in the T-tubule than in fractions SM1 or SM2 (Figure 2b). Immunoelectron microscopy performed on ultrathin sections of extensor digitorum longus muscles also revealed the presence of GLUT4 in T-tubules from rat skeletal muscle (results not shown). However, the difficulty of visualizing long stretches of T-tubules precluded further analysis.

SCAMPs were also present in all cell-surface fractions (Figure 2b). The distribution pattern was different for SCAMP 39 and SCAMP 37. Thus, whereas the highest abundance of SCAMP 39 was detected in fraction SM1, SCAMP 37 was most highly enriched in T-tubule membranes (Figure 2b). Insulin receptors were highly enriched in the T-tubule fraction (Figure 2b). Thus, insulin receptors were 3 times more enriched in T-tubule vesicles than in SM 2 (Figure 2b) and very little insulin receptor was detected in fraction SM1 (Figure 2b).

Additional proof for the presence of GLUT4, insulin receptors and SCAMPs in T-tubules in muscle under basal conditions was obtained by immunoisolation of T-tubule vesicles (Figure 3). In an initial set of experiments, membrane fractions enriched in T-tubules (fraction 23F2) were subjected to immunoisolation by using monoclonal antibody TT-2 that specifically recognizes tt28 protein, a T-tubule marker [31]. Antibody TT-2 immunoadsorbed

Table 1 Abundance of GLUT4 glucose carriers, insulin receptors, SCAMPs and membrane protein markers in different membrane fractions obtained from rat skeletal muscle

The abundance of GLUT4 glucose carriers, insulin receptors, SCAMPs and membrane protein markers in the cell surface was calculated as the percentage of each protein present in a defined fraction in relation to the total amount of protein detected in all fractions (calculated as the sum of all fractions obtained after the sucrose gradient centrifugation). The results are means ± S.E.M. of 4–6 experiments.

	Abundance (% of total protein)							
	Cell-surface membrane	Intracellular membrane fractions			Cell-surface membrane	Intracellular membrane fractions		
	23F1	26F1	29F1	35F1	23F2	26F2	29F2	35F2
GLUT4	10.2 ± 0.6	11.9 ± 1.0	6.3 ± 0.7	3.7 ± 0.6	10.8 ± 0.3	26.4 ± 2.4	15.2 ± 1.5	15.5 ± 0.1
SCAMP 39	8.7 ± 1.2	9.2 ± 3.9	12.8 ± 3.5	10.9 ± 3.6	12.6 ± 1.9	16.3 ± 2.5	15.0 ± 3.7	14.4 ± 2.5
SCAMP 37	4.2 ± 0.9	2.4 ± 0.8	2.7 ± 0.6	5.5 ± 1.7	17.1 ± 2.5	24.7 ± 4.1	23.9 ± 7.2	19.5 ± 5.4
Insulin receptors	2.4 ± 0.1	0.6 ± 0.0	0.6 ± 0.01	1.6 ± 0.6	52.9 ± 2.6	18.9 ± 3.6	6.6 ± 1.2	16.3 ± 2.7
β_1-Integrin	27.3 ± 3.8	6.3 ± 0.3	3.6 ± 0.4	1.9 ± 0.3	39.6 ± 2.3	13.4 ± 0.7	5.9 ± 1.1	1.9 ± 0.9
Dystrophin	1.6 ± 0.7	1.3 ± 0.4	1.0 ± 0.2	0.5 ± 0.3	46.5 ± 4.1	26.4 ± 1.3	13.4 ± 0.9	9.3 ± 4.4
Dihydropyridine receptors	2.8 ± 0.7	0.1 ± 0.0	0.1 ± 0.0	0.7 ± 0.01	72.0 ± 2.4	17.8 ± 0.5	3.6 ± 1.2	2.9 ± 0.3
tt28	2.7 ± 0.2	0.2 ± 0.0	0.1 ± 0.0	0	62.6 ± 2.1	21.2 ± 2.1	9.2 ± 2.6	4.0 ± 0.6

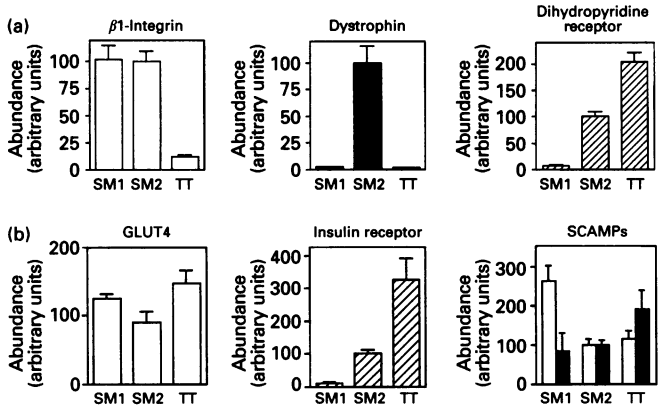

Figure 2 Distribution of GLUT4 glucose carriers, insulin receptors and SCAMPs in different cell-surface membrane fractions from skeletal muscle under fasted conditions

(**a**) The abundance of β_1-integrin, dystrophin and dihydropyridine receptors was assayed in surface membrane fractions sarcolemmal membrane 1 (SM1), sarcolemmal membrane 2 (SM2) and T-tubule membranes (TT). (**b**) GLUT4, insulin receptors and SCAMPs (open bars, SCAMP 39; solid bars, SCAMP 37) were assayed in fractions SM1, SM2 and T-tubule membranes. The distribution of β_1-integrin, dystrophin, dihydropyridine receptors, GLUT4 glucose carriers, insulin receptors and SCAMPs was determined by immunoblot analysis. Equal amounts of membrane proteins from the different fractions were laid on gels. Autoradiograms were subjected to scanning densitometry. The results (means ± S.E.M.) of 4–5 experiments were expressed as arbitrary units. Abundance of GLUT4 in T-tubule membranes was significantly different than in SM1 or SM2 fractions, at $P < 0.05$.

tt28 in a specific and dose-dependent manner (Figure 3). Due to limitations in the supply of antibody TT-2 (the hybridoma cell line is no longer available) we worked under conditions in which the percentage of tt28 being immunoadsorbed was around 20%. T-tubule vesicles immunoisolated with antibody TT-2 also contained dihydropyridine receptors, GLUT4, insulin receptors and SCAMPs (Figures 3 and 4). However, and in keeping with data shown in Figure 1, T-tubule vesicles immunoadsorbed with antibody TT-2 did not contain dystrophin (Figure 3).

In other experiments, vesicles from fraction 23F2 were immunoadsorbed with monoclonal antibody 1F8, which is specific for GLUT4 [24]. Nearly 40% of all GLUT4 found in 23F2 was specifically immunoadsorbed with 1F8 (Figure 4). Under these conditions, tt28, dihydropyridine receptors and insulin receptors were also detected in the immunoadsorbed fraction (Figure 4). Vesicles of intracellular origin (29F2) were also subjected to immunoadsorption with monoclonal antibody 1F8. Under our experimental conditions, approximately 32% of all GLUT4 found in fractions was immunoadsorbed; however, no immunoadsorption of tt28, dihydropyridine receptors, or insulin receptors was detected (Figure 4). Immunoadsorption assays from intracellular membranes revealed a high level of non-specific binding for GLUT4 (Figure 4). This is in agreement with previous findings [12] and it might be due to specific adherent properties of the intracellular GLUT4-containing vesicles.

Insulin alters in an inverse manner the abundance of GLUT4 and insulin receptors in T-tubules

The effect of a supramaximal dose of insulin (30 min after 10 units of insulin/kg body weight, given intravenously) on the muscle distribution of GLUT4 and insulin receptors was next evaluated. Insulin treatment did not alter the protein yield in the different membrane fractions obtained after the processing of skeletal muscle (results not shown). Insulin significantly increased the presence of GLUT4 in T-tubules (64 ± 9% increase) and in fraction SM2 (53 ± 7% increase) (Figure 5). No effect of insulin on GLUT4 was detected in fraction SM1 (Figure 5). This pattern of changes in the distribution of GLUT4 in surface membranes was specific to the glucose transporter, and no effect of insulin was detected in the distribution of the surface markers β_1-integrin, tt28 (Figure 5), dihydropyridine receptors, or dystrophin (results not shown).

Concomitant to the changes observed in cell-surface domains, insulin treatment caused a marked and significant decrease in the content of GLUT4 in intracellular vesicles derived from fractions 26F1, 29F1 and 35F1 (levels in 26F1, 29F1 and 35F1 fractions in the insulin-treated group were 40 ± 5%, 19 ± 2% and 67 ± 12% of values found in the control group) (Figure 5). However, no effect of insulin was detected on the GLUT4 content of intracellular fractions 26F2, 29F2 or 35F2 (Figure 5). Under these conditions, the total amount of GLUT4 recovered from all

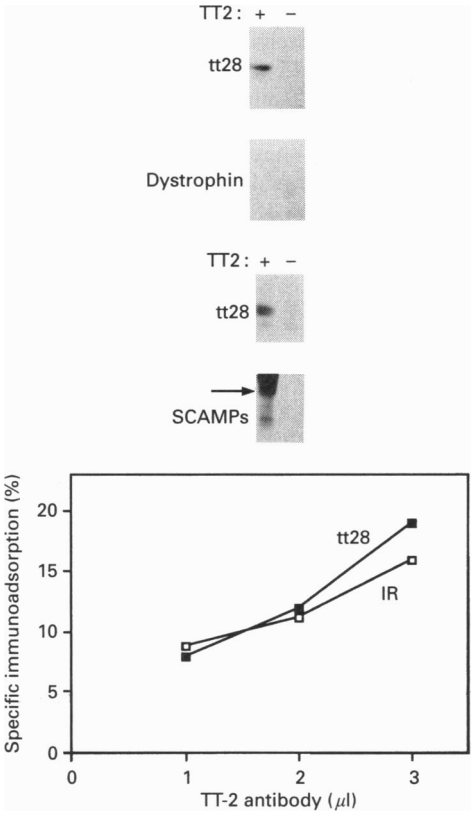

Figure 3 Immunoadsorption of tt28-containing T-tubule vesicles

Insulin receptor and SCAMPs, but not dystrophin, co-localize with T-tubule membranes. Membrane fractions enriched in T-tubules (fraction 23F2) obtained from skeletal muscle were incubated with or without immobilized TT-2 antibodies. After the incubation, the adsorbed fractions were electrophoresed and immunoblotted to determine the abundance of tt28 protein, insulin receptors (IR), SCAMPs or dystrophin. Autoradiographs were subjected to scanning densitometry. Representative autoradiograms, obtained after various times of exposure, are shown (top and middle panels). The arrow in the middle panel indicates the presence of the immunoglobulins. In some experiments, membrane vesicles (23F2) were incubated with increasing amounts of immobilized antibody TT-2. After the incubation, the adsorbed fractions were electrophoresed and immunoblotted to determine the abundance of the tt28 protein and the insulin receptor. Autoradiographs were subjected to scanning densitometry. Results (means ± S.E.M.) of 5–7 experiments after vesicle immunoisolation were expressed as a percentage of specific immunoadsorption (bottom panel).

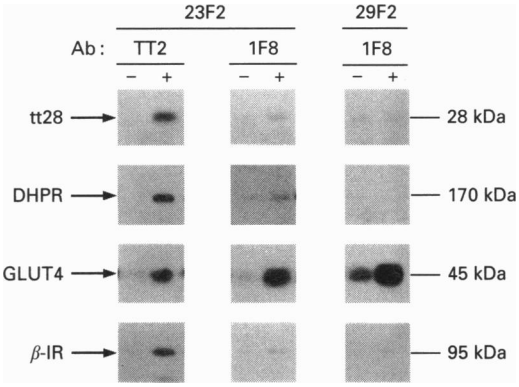

Figure 4 Co-localization of GLUT4, insulin receptors, tt28 and dihydropyridine receptors after immunoadsorption of T-tubule fractions with immobilized antibody TT-2 or 1F8

Membrane fractions enriched in T-tubules (fraction 23F2) obtained from skeletal muscle were incubated with or without immobilized antibodies TT-2 or 1F8. In some experiments, intracellular membranes (fraction 29F2) were also incubated with or without immobilized antibody 1F8. After the incubation, the adsorbed fractions were electrophoresed and immunoblotted to determine the abundance of tt28 protein, dihydropyridine receptors (DHPR), GLUT4 and insulin receptors (IR). Autoradiograms were subjected to scanning densitometry. Representative autoradiograms, obtained after various times of exposure, are shown.

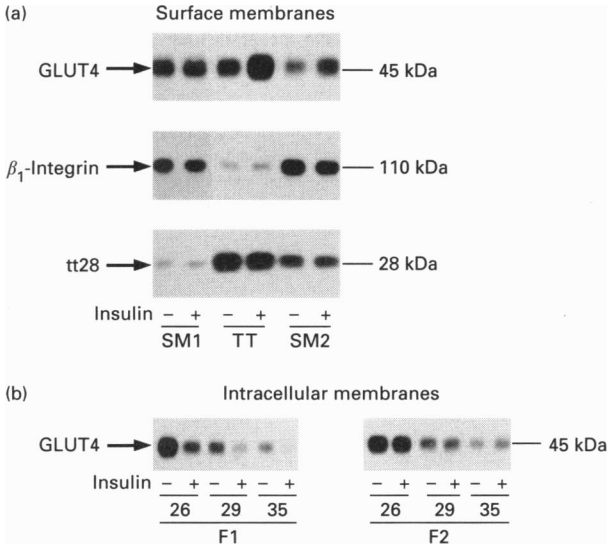

Figure 5 Insulin recruits GLUT4 from intracellular membranes to T-tubules and to sarcolemma in skeletal muscle

The abundance of GLUT4 was assayed in surface membrane fractions SM1, TT and SM2 (**a**) and in intracellular membranes 26F1, 29F1, 35F1, 26F2, 29F2 and 35F2 (**b**) from control and insulin-stimulated muscles. β_1-Integrin and tt28 were also assayed in surface membrane fractions SM1, TT and SM2 (**a**). The distribution of the GLUT4, β1-integrin and tt28 was determined by immunoblot analysis by using specific antibodies (see the Materials and methods section). Equal amounts of membrane proteins (1 μg) from the different fractions were laid on gels. Representative autoradiograms from 4–7 experiments are shown.

fractions was not different in control and insulin-treated muscles (results not shown).

Additional evidence for an insulin-induced translocation of GLUT4 to T-tubules came from immunoadsorption experiments. 23F2 muscle fractions from non-stimulated and insulin-treated rats were subjected to vesicle immunoisolation using immobilized monoclonal antibody TT-2. Antibody TT-2 immunoadsorbed similar amounts of tt28 in control and insulin-treated groups (Figure 6). Under these conditions, antibody TT-2 immunoadsorbed 1.8-fold more GLUT4 in the insulin-treated group than in the control group (Figure 6).

Insulin treatment *in vivo* also caused a substantial alteration in the distribution of insulin receptors in muscle membrane fractions (Figure 7). Thus, the presence of insulin receptors was markedly decreased in the T-tubule fraction (42 ± 6%) and in fraction SM2 (54 ± 6%) (Figure 7). Under these conditions, the presence of insulin receptors was markedly enhanced by insulin (92 ± 15% increase) in the intracellular fraction 35F2 (Figure 7). Insulin did not modify the total amount of insulin receptors recovered in fractions (results not shown).

Insulin redistributes SCAMPs in the muscle fibre

Insulin causes some redistribution of SCAMPs from low-density microsomes to the plasma membrane in isolated rat adipocytes

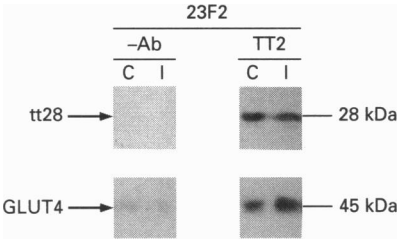

Figure 6 Insulin increases GLUT4 abundance in T-tubule membranes immunoadsorbed with immobilized antibody TT-2

Membrane fractions enriched in T-tubules (fraction 23F2) obtained from non-stimulated (C) and insulin-stimulated (I) skeletal muscle were incubated without (— Ab) or with (TT-2) immobilized antibody TT-2. After the incubation, the adsorbed fractions were electrophoresed and immunoblotted to determine the abundance of GLUT4 and tt28 protein. Autoradiograms were subjected to scanning densitometry. Representative autoradiograms, obtained after various times of exposure, from four separate experiments are shown.

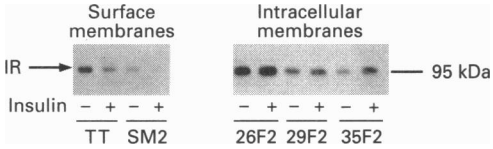

Figure 7 Insulin internalizes insulin receptors from T-tubules and sarcolemma to intracellular membranes in skeletal muscle

The abundance of insulin receptors was assayed in surface membrane, SM2 and TT and in intracellular membranes 26F2, 29F2 and 35F2 from control and insulin-stimulated muscles. The distribution of insulin receptors was determined by immunoblot analysis. Equal amounts of membrane proteins (4 μg) from the different fractions were laid on gels. Autoradiograms were subjected to scanning densitometry. Representative autoradiograms from four to five experiments are shown.

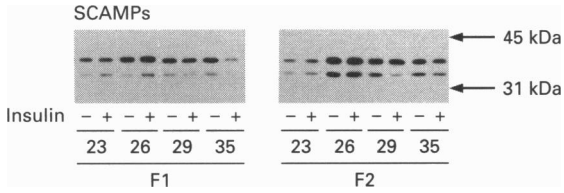

Figure 8 Insulin redistributes SCAMPs in membrane fractions from skeletal muscle

The abundance of SCAMPs was assayed in cell-surface membrane fractions 23F1 and 23F2 and in intracellular membranes 26F1, 29F1, 35F1, 26F2, 29F2 and 35F2 from control and insulin-stimulated muscles. The distribution of the SCAMPs was determined by immunoblot analysis by using antibody 3F8. Equal amounts of membrane proteins (1 μg) from the different fractions were laid on gels. Representative autoradiograms from 6–9 experiments are shown.

[27,39]. To determine whether insulin also affects the distribution of SCAMPs in the muscle fibre and, if so, whether T-tubule membranes are affected, the abundances of SCAMP 39 and SCAMP 37 in intracellular and cell-surface membranes before and after insulin treatment were next studied (Figure 8). The abundance of both SCAMP 39 and SCAMP 37 was increased in response to insulin in membrane fractions 23F1 (enriched in sarcolemmal membranes) and 26F1 (an intracellular membrane fraction) (levels of SCAMP 39 in the insulin-treated group increased by 26 ± 4 % and 25 ± 9 % compared with control values in 23F1 and 26F1 respectively); levels of SCAMP 37 increased in response to insulin (95 ± 23 % and 63 ± 18 % in 23F1 and 26F1 respectively) (Figure 8). Under these conditions, the levels of SCAMP 39 and SCAMP 37 were markedly decreased in the insulin-treated group in intracellular fractions 29F1 and 35F1 (levels of SCAMP 39 decreased in response to insulin by 54 ± 18 % and 49 ± 15 % in 29F1 and 35F1 respectively; levels of SCAMP 37 decreased in response to insulin by 37 ± 12 % and 65 ± 23 % in 29F1 and 35F1 respectively) (Figure 8). Furthermore, insulin enhanced the presence of SCAMP 37 in fraction 23F2 (enriched in membranes from sarcolemma and T-tubule) and decreased the content of SCAMP 37 in fractions 29F2 and 35F2, both of intracellular origin (Figure 8). These data suggest that insulin redistributes SCAMPs in a complex manner from some intracellular membrane fractions to other intracellular membranes as well as to the cell surface. However, no increase in SCAMP abundance was observed in T-tubule membranes after insulin treatment (results not shown).

DISCUSSION

The purpose of this study was to perform a highly detailed analysis of the subcellular distribution of certain membrane proteins known to be involved in insulin action in the muscle fibre, namely GLUT4 glucose carriers, insulin receptors and SCAMPs. Under fasting basal conditions, each of these membrane proteins was distributed in a unique manner. Thus, insulin receptors were mainly found in the cell surface; they were highly abundant in T-tubules and they were also present in sarcolemmal domain 2 (SM2), but were virtually absent from sarcolemmal domain 1 (SM1). In contrast, most GLUT4 carriers and SCAMPs were detected intracellularly under fasting conditions. In addition, GLUT4 and SCAMPs were found both in T-tubules and in sarcolemma, each with a distinct distribution pattern. Whereas GLUT4 and SCAMP 37 showed their greatest abundance in T-tubules, SCAMP 39 was most abundant in sarcolemmal membrane fraction 1 (SM1).

Under basal conditions (low insulin levels), GLUT4 glucose carriers, insulin receptors and SCAMPs are present in T-tubules. This conclusion is based on the following findings: (a) GLUT4 has been detected in T-tubules by immunogold analysis [8,11]; (b) GLUT4 and insulin receptors have been detected in highly purified T-tubule membranes [9,40]; (c) GLUT4, insulin receptors and SCAMPs have been detected in highly purified T-tubule membranes free from sarcolemmal components ([21] and this study); and (d) there is co-localization of GLUT4, insulin receptors, SCAMPs and T-tubule markers (protein tt28 and dihydropyridine receptors) after immunoadsorption of T-tubule vesicles either with monoclonal antibody TT-2 directed against tt28 or with monoclonal antibody 1F8 against GLUT4 (this study).

Our data indicate that under basal conditions, GLUT4, SCAMPs and insulin receptors are distributed in the cell surface according to different distribution patterns. Thus, whereas GLUT4, SCAMP 37 and insulin receptors were more abundant in T-tubules than in the sarcolemma, SCAMP 39 abundance was greater in sarcolemma than in T-tubules. These results suggest a complex set of exocytic and endocytic processes controlling the distribution of GLUT4 glucose carriers, SCAMPs and insulin receptors in different cell-surface domains of the muscle fibre. SCAMPs are proposed to be markers for general cell-surface recycling systems [23], but their precise function remains unknown. The differential abundance of SCAMP 39 and SCAMP

37 in different domains of the sarcolemma and in T-tubules suggests the existence of separate membrane protein recycling systems in the muscle fibre which possess different proportions of each SCAMP.

In response to acute insulin treatment, GLUT4 in the muscle fibre was translocated to selective domains of the sarcolemma and also to T-tubules. Based on the fact that insulin recruits GLUT4 to a dystrophin-enriched sarcolemmal fraction, and based on the selective distribution of dystrophin in sarcolemma [41], we suggest that this is a costameric sarcolemmal domain. These results indicate that both sarcolemma and T-tubules constitute important target domains of insulin action. This finding poses the question as to where insulin action is initiated in the muscle fibre. In this regard, we found that insulin-induced GLUT4 recruitment occurs in those cell-surface domains containing insulin receptors, which suggests that both sarcolemma and T-tubules are also target domains regarding the initiation of insulin action in the muscle fibre.

The insulin-dependent translocation of GLUT4 to T-tubules was substantiated by the following observations: (a) GLUT4 abundance increased in membrane preparations highly enriched in T-tubules that are devoid of sarcolemmal vesicles; and (b) immunoadsorption of T-tubule vesicles with antibody TT-2 led to greater recovery of GLUT4 in the immunoprecipitates from the insulin-treated group than in those from the control group. Our data indicating insulin-induced GLUT4 recruitment to the T-tubules of the muscle fibre extend prior observations performed in skeletal muscle by immunoelectron microscopy [8], 2-*N*-4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis-(D-mannos-4-yloxy)-2-propylamine (ATB-[2-^{3}H]BMPA) photolabelling coupled to autoradiography [10], or subcellular fractionation analysis [9]. The uptake of glucose through the T-tubule membrane after insulin stimulation offers the advantage of a direct channelling of glucose to hexokinase bound to the outer mitochondrial membrane near the T-tubules [17–19], and then to the rest of the glycolytic enzymes, also located in the vicinity of T-tubules [14–16].

After insulin treatment, the abundance of GLUT4 expressed per μg of protein in T-tubules was 1.8-fold greater than in sarcolemma. Available morphometric measurements in fast-twitch glycolytic and fast-twitch oxidative muscles indicate that the surface area of T-tubules is 1.5-fold greater than the surface area of sarcolemma [32]. Linking these two items of information, we propose that the process of GLUT4 translocation towards the T-tubule might be predominant compared with the translocation towards the sarcolemma in fast-twitch muscle fibres. The situation might be somewhat different in slow-twitch red fibres (i.e. soleus muscle), which are characterized by a lower density of T-tubules [32].

In response to acute insulin administration, the abundance of insulin receptors decreased by 44% in both T-tubule and sarcolemmal fractions concomitant with a corresponding increase in insulin receptors in a high-density intracellular membrane fraction (fraction 35F2). These results demonstrate that insulin receptors from both T-tubules and sarcolemma undergo insulin-induced internalization. This is the expected result based on numerous studies fron various cells, although no information was available in muscle [42–44]. The current study is unique, however, in demonstrating that insulin-mediated insulin receptor internalization occurs from both T-tubule and sarcolemma fractions. Consequently, these results indicate that the insulin receptors from all domains of the cell surface appear to be fully active in transducing insulin action in the rat muscle fibre.

In contrast to the distinct pattern of insulin-induced redistribution of GLUT4 and insulin receptors in the muscle fibre, SCAMPs showed insulin-induced redistribution different from that found for GLUT4 and insulin receptors. SCAMPs moved in response to insulin from intracellular high-density fractions to intracellular lighter-density fractions and to the cell surface, showing a distinct pattern of insulin-induced cellular redistribution. However, under these conditions, no increase in SCAMPs was detected in T-tubules after insulin treatment. These results suggest that SCAMPs redistribute in a more complex manner than GLUT4. In the absence of a defined function for these proteins, nothing further can be concluded concerning them.

We found that concomitant with GLUT4 recruitment to sarcolemma and T-tubules, insulin treatment causes a marked decrease in the GLUT4 content of certain intracellular vesicles. The intracellular membrane fractions originating from the low-speed homogenization step (F1), representing 28% of the total intracellular GLUT4 pool, are substantially depleted after insulin treatment. However, the intracellular membrane fractions originating from the high-speed homogenization step (F2), representing 72% of the total intracellular GLUT4 pool, do not change in response to insulin. In summary, these findings provide evidence for the existence of at least two different pools of intracellular GLUT4 hexose carriers in skeletal muscle. Our results are consistent with the existence of different intracellular GLUT4 pools suggested previously on the basis of the identification of an intracellular pool of GLUT4 vesicles obtained from rat skeletal muscle that becomes GLUT4-depleted after insulin treatment but not by acute exercise [45,46]. Our results show that approximately 12% of the intracellular GLUT4 was redistributed to the cell surface. These observations substantiate the existence of a large intracellular reservoir of GLUT4 compared with the requirements of insulin action in skeletal muscle.

Current available information regarding insulin-induced GLUT4 translocation in muscle fibre can be summarized according to the following model. Under fasted conditions where insulin levels are low, most GLUT4 is intracellular in the vicinity of Golgi, T-tubules and sarcolemma. Insulin causes the translocation of GLUT4 to T-tubules as well as to selective domains of the sarcolemma–costameric sarcolemmal domain, and the cell-surface domains affected by GLUT4 recruitment also contain insulin receptors. Redistribution of intracellular GLUT4 affects a rather small percentage of the total intracellular GLUT4. In this regard, we have identified two different intracellular GLUT4 populations: an insulin-sensitive pool that is depleted in response to insulin treatment and an insulin-insensitive pool. The biochemical characterization of these membrane populations might be fundamental in the understanding of the processes triggered by insulin that lead to the recruitment of GLUT4 to the cell surface of the muscle fibre.

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