Isolation and characterization of distinct domains of sarcolemma and T-tubules from rat skeletal muscle

Purificación MUÑOZ,* Mario ROSEMBLATT,† Xavier TESTAR,* Manuel PALACÍN* and Antonio ZORZANO*‡

*Departament de Bioquímica i Fisiologia, Facultat de Biologia, Universitat de Barcelona, Avda. Diagonal 645, 08028 Barcelona, Spain and †Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Santiago, Chile

1. Several cell-surface domains of sarcolemma and T-tubule from skeletal-muscle fibre were isolated and characterized. 2. A protocol of subcellular fractionation was set up that involved the sequential low- and high-speed homogenization of rat skeletal muscle followed by KCl washing, Ca^{2+} loading and sucrose-density-gradient centrifugation. This protocol led to the separation of cell-surface membranes from membranes enriched in sarcoplasmic reticulum and intracellular GLUT4-containing vesicles. 3. Agglutination of cell-surface membranes using wheat-germ agglutinin allowed the isolation of three distinct cell-surface membrane domains: sarcolemmal fraction 1 (SM1), sarcolemmal fraction 2 (SM2) and a T-tubule fraction enriched in protein tt28 and the α_2-component of dihydropyridine receptor. 4. Fractions SM1 and SM2 represented distinct sarcolemmal subcompartments based on different compositions of biochemical markers: SM2 was characterized by high levels of β_1-integrin and dystrophin, and SM1 was enriched in β_1-integrin but lacked dystrophin. 5. The caveolae-associated molecule caveolin was very abundant in SM1, SM2 and T-tubules, suggesting the presence of caveolae or caveolin-rich domains in these cell-surface membrane domains. In contrast, clathrin heavy chain was abundant in SM1 and T-tubules, but only trace levels were detected in SM2. 6. Immunoadsorption of T-tubule vesicles with antibodies against protein tt28 and against GLUT4 revealed the presence of GLUT4 in T-tubules under basal conditions and it also allowed the identification of two distinct pools of T-tubules showing different contents of tt28 and dihydropyridine receptors. 7. Our data on distribution of clathrin and dystrophin reveal the existence of subcompartments in sarcolemma from muscle fibre, featuring selective mutually exclusive components. T-tubules contain caveolin and clathrin suggesting that they contain caveolin- and clathrin-rich domains. Furthermore, evidence for the heterogeneous distribution of membrane proteins in T-tubules is also presented.

INTRODUCTION

Glucose transport in muscle is mainly catalysed by the GLUT4 glucose-carrier isoform, and its activity is acutely regulated by a variety of factors such as hormones and the level of contractile activity [1]. Our understanding of the cell biology of glucose carriers in muscle fibre is hampered by major technical difficulties associated with the isolation of distinct membrane fractions.

Muscle fibre is covered by a membrane called the surface sarcolemma. The sarcolemma is a multicomponent structure that can be divided into the innermost component, the plasma membrane and an overlying basement membrane consisting of an inner basal lamina and an external reticular lamina [2]. The sarcolemma contains membrane proteins that are involved in the transport of ions and organic substrates, such as Na^+/K^+-ATPase and glucose carriers, and also membrane proteins that are involved in signalling, such as insulin receptors and β-adrenergic receptors [3–7]. It has recently been found that the location of certain proteins such as β_1-integrin, dystrophin and dystrophin-associated glycoproteins is restricted to the sarcolemma of muscle fibre [8–13]. Morphological observations have also revealed the presence of invaginating caveolae in the sarcolemma [14,15] which can be clustered in groups of two or three, connecting to the extracellular space through a common neck.

Structurally continuous with the surface sarcolemma, and invaginating into the muscle fibre, is the T-tubule membrane system. In addition to providing access of extracellular fluid to the interior of the muscle cell, an important physiological function of the T-tubule system is the transmission of membrane depolarization to the central part of the muscle to activate contraction by causing Ca^{2+} release by the sarcoplasmic reticulum. T-tubules consist of free and junctional regions, and the proportion of junctional T-tubules is higher in fast-contracting than in slow fibres [16]. The protein composition of the T-tubule membrane reflects its dual role in excitability and signal transduction. T-tubules express proteins shared with the plasma membrane, such as the voltage-gated Na^+ channel, the α_1 isoform of Na^+/K^+-ATPase, β-adrenergic receptors and G-proteins [7,17–20], as well as a distinct set of membrane proteins, some of which, such as the dihydropyridine receptor, are involved in excitation–concentration coupling [21–25].

Based on our interest in the cell-surface components that contain glucose carriers, we here report the isolation and characterization of different cell-surface domains of sarcolemma and T-tubule from the muscle fibre of the rat. Our data reveal the existence of subcompartments in the sarcolemma, featuring various types of component some of which are mutually exclusive. Furthermore, evidence for the heterogeneous distribution of membrane proteins in T-tubules is also presented.

MATERIALS AND METHODS

Materials

^{125}I-Protein A and ^{125}I-labelled sheep anti-mouse antibody were purchased from ICN. Immobilon was obtained from Millipore. All electrophoresis reagents and molecular-mass markers were obtained from Bio-Rad. γ-Globulin and most commonly used chemicals were from Sigma.

Abbreviation used: WGA, wheat-germ agglutinin.

‡ To whom correspondence should be addressed.

Antibodies

Both monoclonal (1F8) and polyclonal (OSCRX) antibodies specific for GLUT4 were used in these studies. Monoclonal antibody 1F8 [26] was generously given by Dr. Paul F. Pilch (Boston University, Boston, MA, U.S.A.). Anti-GLUT4 (OSCRX) from rabbit was produced after immunization with a peptide corresponding to the final 15 amino acids of the C-terminus [27]. A rabbit polyclonal antibody against rat β_1-integrin was generously provided by Dr. Carles Enrich (Universitat de 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 purchased from Novocastra. Monoclonal antibody SY38 against synaptophysin was from Boehringer-Mannheim. Monoclonal antibody A-52 against Ca^{2+}-ATPase [30] was donated by Dr. David H. MacLennan (University of Toronto, Toronto, Ont., Canada). Polyclonal antibody against clathrin heavy chain was obtained from ICN Immunobiologicals, and polyclonal antibody against caveolin was given by Dr. Senen Vilaró (Universitat de Barcelona). Monoclonal antibody TT-2, which recognizes protein tt28, specific for T-tubules [24] was also used in these studies.

Animals and tissue sampling

Male Wistar rats weighing between 250 and 300 g from the colony of the Universitat de Barcelona 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 anaesthesized with sodium pentobarbital, and white portions of gastrocnemius and quadriceps muscles were rapidly excised and immediately processed. Muscle enriched in white fibres was used because of its greater content of T-tubule membranes [31].

Isolation of rat skeletal-muscle membranes

The different cell-surface and intracellular membrane fractions were isolated as follows (see Figure 1). Approx. 12 g of rat skeletal muscle was excised, weighed, minced and initially homogenized in a Polytron homogenizer at low speed (setting 4; 2 × 5 s) in buffer A (20 mM Tris/HCl, pH 7.4, 0.25 M sucrose, 1 mM EDTA, 1 μM pepstatin, 1 μM leupeptin; 1 g/4 ml). The homogenate was centrifuged for 20 min at 10000 *g* (9000 rev./min in an SA-600 Sorvall rotor). 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 designated fraction F1. The pellet was resuspended in buffer A and subjected to 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 designated F2. Fractions F1 and F2 were incubated with 0.6 M KCl for 1 h at 4 °C and then pelleted for 1 h at 150000 *g* in a T-647.5 Sorvall rotor. The pellets from KCl-washed fractions F1 and F2 were then subjected to Ca^{2+} loading in order to increase the density of sarcoplasmic-reticulum vesicles [32]. To this end, pellets were resuspended in buffer B (50 mM potassium phosphate, 4 mM $MgCl_2$, 150 mM KCl, pH 7.5) at a protein concentration of 2 mg/ml. Ca^{2+} loading was initiated by addition of 0.3 mM $CaCl_2$ and 2 mM ATP. After incubation for 20 min at room temperature, fractions F1 and F2 were kept on ice and centrifuged for 60 min at 150000 *g*. 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* (25000 rev./min in a TH-641 Sorvall rotor), four protein fractions were separated from the 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. Proteins were determined by the method of Bradford [33] using γ-globulin as 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 wheatgerm agglutinin (WGA) [12,34]. 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 WGA (Sigma) in buffer D. The total volume of this mixture was 600 μl and after 10 min of 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 deagglutinated by incubation for 20 min at 0 °C in 500 μl of 0.3 M *N*-acetyl-D-glucosamine in buffer E. The deagglutinated suspension was centrifuged in a Microfuge for 1.5 min at 15000 *g* and the supernatant was centrifuged 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. Starting with 100 μg of 23F1 membrane proteins, the yield of purified sarcolemmal fraction 1 (23F1 W^+) was about 35 μg. Starting with 100 μg of 23F2 membrane proteins, the yields of purified sarcolemmal fraction 2 (23F2 W^+) and 23F2 W^- were about 31 μg and 11 μg respectively. (See the Results section for definitions of these fractions.)

Protocols of T-tubule vesicle immunoisolation

Antibodies 1F8 (4–5 μg) and TT-2 (1–3 μl) were incubated overnight at 4 °C with goat anti-mouse IgG coupled to agarose (75 μl bead volume) (Sigma). Beads were collected by a 6 s spin in a Microfuge and washed in PBS. Intact membrane preparations (15–25 μg of proteins of 23F2) 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 of membrane proteins was performed as described by Laemmli [35]. For GLUT4, dihydropyridine receptors, Ca^{2+}-

ATPase, β_1-integrin, tt28, synaptophysin and caveolin, non-reducing 8.5 or 12% polyacrylamide gels were used. For immunodetection of dystrophin and clathrin heavy chain, samples were reduced with 100 mM dithiothreitol and run in 6% gels. Proteins were transferred to Immunobilon as previously reported [36] in buffer consisting of 20% methanol, 200 mM glycine and 25 mM Tris/HCl, pH 8.3. After transfer, the filters were blocked with 5% non-fat dry milk/0.02% sodium azide in PBS for 1 h at 37 °C and then 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 sheep anti-mouse ^{125}I-antibody. The autoradiograms were quantified using scanning densitometry. Immunoblots were performed under conditions in which autoradiographic detection was in the linear response range.

Table 1 Protein yields in membrane fractions

Results are means ± S.E.M. (n = 6–8 for each group). KCl-washed Ca^{2+}-loaded fractions F1 and F2 refer to the fractions that were loaded on the sucrose-density gradients. The mean body weight of the rats was 266 ± 5 g and the mean muscle weight 12.9 ± 0.3 g.

Fraction	Total protein (mg)
KCl-washed Ca^{2+}-loaded F1	9.0 ± 0.5
23F1	0.324 ± 0.051
26F1	0.135 ± 0.024
29F1	0.185 ± 0.031
35F1	0.278 ± 0.022
Pellet-F1	2.119 ± 0.207
KCl-washed Ca^{2+}-loaded F2	21.2 ± 2.2
23F2	0.518 ± 0.078
26F2	0.289 ± 0.056
29F2	0.506 ± 0.048
35F2	1.157 ± 0.131
Pellet-F2	8.733 ± 0.299

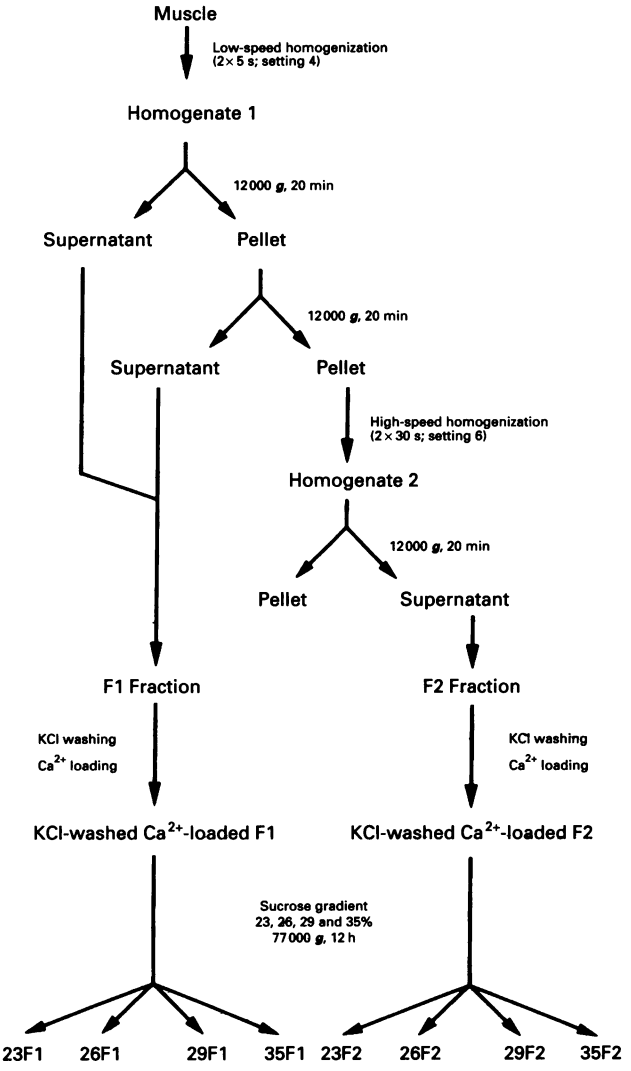

Figure 1 Flow chart of procedure used to isolate different membrane fractions from rat skeletal muscle

RESULTS

Isolation of membrane fractions enriched in cell-surface domains

In order to isolate different cell-surface domains of the muscle fibre in rat skeletal muscle, we set up a protocol that involved obtaining two crude membrane fractions by sequential homogenization at low speed (F1) and high speed (F2) starting from a single preparation of muscle tissue. F1 and F2 fractions were subjected to KCl washing, Ca^{2+} loading and centrifugation in a discontinuous sucrose-density gradient. Different fractions were finally obtained from F1 (23F1, 26F1, 29F1, 35F1 and pellet-F1) and F2 (23F2, 26F2, 29F2, 35F2 and pellet-F2) (Figure 1, Table 1). These membrane fractions were characterized on the basis of the abundance of markers for sarcolemmal membrane (β_1-integrin and dystrophin) [8–11] and T-tubule membrane (α_2-component of dihydropyridine receptors and tt28 protein) [23,37,38]. Fractions obtained from the top of the sucrose gradients (23F1 and 23F2) were maximally enriched in sarcolemmal and T-tubule markers (Figure 2). 23F1 membranes were highly enriched in β_1-integrin (59-fold enrichment over KCl-washed Ca^{2+}-loaded F1 fraction) and showed very low abundance of dystrophin, the α_2-component of dihydropyridine receptors and tt28 protein (Figure 2). In contrast, 23F2 membranes were enriched in all cell-surface markers analysed: β_1-integrin (44-fold enrichment over KCl-washed Ca^{2+}-loaded F2 fraction), dystrophin, dihydropyridine receptors (54-fold enrichment over KCl-washed Ca^{2+}-loaded F2 fraction) and tt28 (60-fold enrichment over KCl-washed Ca^{2+}-loaded F2 fraction) (Figure 2).

23F1 and 23F2 fractions contained most of the sarcolemmal and T-tubule markers. Thus the total content of β_1-integrin in the 23F1 fraction accounted for 27% of the β_1-integrin recovered in all fractions (legend to Figure 2); in addition, the content of β_1-integrin, dystrophin, dihydropyridine receptors and tt28 found in the 23F2 fraction was 40, 47, 72 and 63% of total recovered (legend to Figure 2).

23F1 and 23F2 fractions were essentially free of Ca^{2+}-ATPase, sarcoplasmic-reticulum marker (Figure 3); Ca^{2+}-ATPase was abundant in the 35F1 and 35F2 fractions as well as in pellet-F1 and pellet-F2. When the abundance of Ca^{2+}-ATPase was expressed per total fraction, the content of this marker in 35F2, pellet-F2 and pellet-F1 accounted for 9, 62 and 20% of all Ca^{2+}-ATPase recovered in the fractions (legend to Figure 3). The abundance of GLUT4 in the membrane fractions derived from

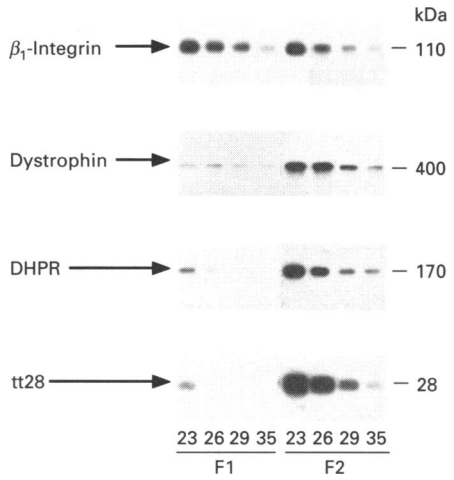

Figure 2 Demonstration that membrane fractions 23F1 and 23F2 are enriched in sarcolemmal and T-tubule markers

The abundance of β_1-integrin, dystrophin, the α_2-component of dihydropyridine receptors and tt28 protein was assayed in fractions 23F1, 26F1, 29F1, 35F1 and 23F2, 26F2, 29F2, 35F2 obtained by sucrose-density-gradient centrifugation of F1 and F2 membrane preparations. The distribution of the different markers was studied by immunoblot analysis using specific antibodies (see the Materials and methods section). Equal amounts of membrane protein (1–3 μg) from the different fractions were loaded on the gels. Autoradiograms were subjected to scanning densitometry. Data were expressed as the percentage that each fraction contributed to the total amount of marker detected in all fractions. Values (means ± S.E.M. of four to six experiments) for β_1-integrin were 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 and 1.9 ± 0.9% in fractions 23F1, 26F1, 29F1, 35F1, 23F2, 26F2, 29F2 and 35F2 respectively. Values for dystrophin were 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 and 9.3 ± 4.4% in fractions 23F1, 26F1, 29F1, 35F1, 23F2, 26F2, 29F2 and 35F2 respectively. Values for the α_2-component of dihydropyridine receptors (DHPR) were 2.8 ± 0.7, 0.2 ± 0.01, 0.1 ± 0.01, 0.7 ± 0.01, 72.0 ± 2.4, 17.8 ± 0.5, 3.6 ± 1.2 and 2.9 ± 0.2% in fractions 23F1, 26F1, 29F1, 35F1, 23F2, 26F2, 29F2 and 35F2 respectively. Values for tt28 protein were 2.7 ± 0.2, 0.2 ± 0.01, 0.1 ± 0.01, 0, 62.6 ± 2.1, 21.2 ± 2.1, 9.2 ± 2.6 and 4.0 ± 0.4 in fractions 23F1, 26F1, 29F1, 35F1, 23F2, 26F2, 29F2 and 35F2 respectively.

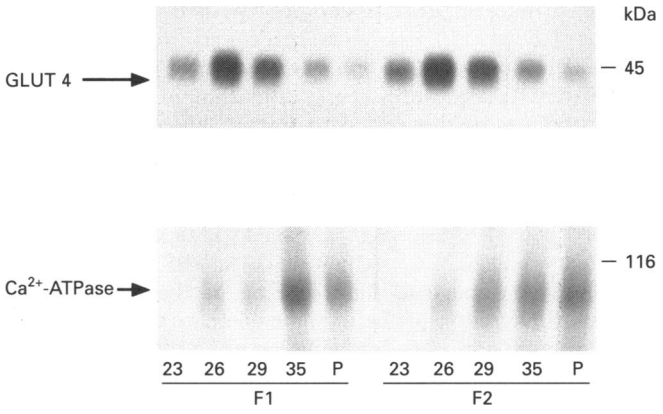

Figure 3 Abundance of the intracellular markers Ca^{2+}-ATPase and GLUT4 in membrane fractions obtained by sucrose-density-gradient centrifugation of F1 and F2 preparations

The abundance of Ca^{2+}-ATPase and GLUT4 was assayed in fractions 23F1, 26F1, 29F1, 35F1, pellet-F1 (P) and 23F2, 26F2, 29F2, 35F2 and pellet-F2 (P) obtained by sucrose-density-gradient centrifugation of F1 and F2 membrane preparations. The distribution of these markers was studied by immunoblot analysis using specific antibodies (see the Materials and methods section). Equal amounts of membrane protein (1–3 μg) from the different fractions were loaded on the gels. Autoradiograms were subjected to scanning densitometry. Data were expressed as the percentage that each fraction contributed to the total amount of GLUT4 or Ca^{2+}-ATPase detected in all fractions. Values for Ca^{2+}-ATPase (means ± S.E.M. of four to six experiments) were 0.2 ± 0.05, 0.1 ± 0.06, 0.9 ± 0.4, 2.4 ± 0.9, 19.9 ± 3.4, 0.2 ± 0.05, 0.4 ± 0.2, 5.1 ± 1.4, 8.6 ± 0.5 and 62.2 ± 9.2% in fractions 23F1, 26F1, 29F1, 35F1, pellet-F1, 23F2, 26F2, 29F2, 35F2 and pellet-F2 respectively. Values for GLUT4 were 7.6 ± 0.4, 11.5 ± 1.0, 6.3 ± 0.7, 3.3 ± 0.5, 1.3 ± 0.1, 9.7 ± 0.3, 23.7 ± 2.1, 13.6 ± 1.3, 13.9 ± 2.1 and 9.2 ± 0.8% in fractions 23F1, 26F1, 29F1, 35F1, pellet-F1, 23F2, 26F2, 35F2 and pellet-F2 respectively.

F1 and F2 showed a different pattern from that found for cell-surface markers or sarcoplasmic reticulum. Thus the greatest abundance of GLUT4 was found in fractions 26F1 (455% of 23F2) and 26F2 (436% of 23F2); high levels were also found in fractions 29F1 (levels accounted for 181% of 23F2) and 29F2 (143% of 23F2) (Figure 3). When the data were expressed as GLUT4 content in the total fraction, we found that the richest source of GLUT4 was fraction 26F2 (24%) (legend to Figure 3), whereas cell-surface fractions, i.e. 23F1 and 23F2, only accounted for 8 and 10% of GLUT4 recovered in all fractions (legend to Figure 3).

Our data indicate that sequential low- and high-speed homogenization of rat skeletal muscle followed by KCl washing, Ca^{2+} loading and sucrose-density-gradient centrifugation gives two distinct surface-membrane populations, 23F1 and 23F2. 23F1 contains membranes of sarcolemmal origin and 23F2 contains both sarcolemmal and T-tubule membranes. These cell-surface fractions were separated from membranes of intracellular origin, such as those from GLUT4-containing vesicles or sarcoplasmic reticulum.

Isolation of membranes specifically enriched in sarcolemmal or T-tubule markers by agglutination

To purify 23F1 and 23F2 membranes, fractions were treated with WGA. According to previous studies [12,34], WGA agglutination allows the separation of sarcolemmal vesicles (which are agglutinated with WGA) from T-tubule vesicles (which are not agglutinated with WGA). Treatment of 23F1 vesicles with WGA led to the recovery of an agglutinated fraction (23F1 W^+), whereas no detectable protein was recovered in the non-agglutinated fraction (results not shown). 23F1 W^+ showed a greater abundance of β_1-integrin than fraction 23F1 (results not shown). The high abundance of β_1-integrin and the lack of T-tubule markers is consistent with the view that 23F1 and 23F1 W^+ are fractions of sarcolemmal origin that are T-tubule-free. On the basis of these observations, from here on we refer to 23F1 W^+ as sarcolemmal fraction 1 (SM1), characterized by a high abundance of β_1-integrin and no dystrophin.

Treatment of fraction 23F2 with WGA produced two different fractions, an agglutinatable fraction (23F2 W^+) and a non-agglutinatable fraction (23F2 W^-). These two fractions accounted for 74 and 26% respectively of the total protein recovered after WGA treatment. More importantly, the two fractions differed markedly in protein composition (Figure 4). Thus 23F2 W^+ showed a great enrichment of the sarcolemmal markers β_1-integrin (175% of 23F2) and dystrophin (288% of 23F2) and no enrichment of the T-tubule markers, dihydropyridine receptors (96% of 23F2) and tt28 (86% of 23F2). These properties allowed us to define fraction 23F2 W^+ as sarcolemmal fraction 2 (SM2), which is clearly different from SM1 (i.e. 23F1 W^+) (Figure 5). On the other hand, 23F2 W^- had a greater enrichment of the T-tubule markers, dihydropyridine receptors (195% of 23F2) and tt28 protein (187% of 23F2) (Figure 4), and the sarcolemmal markers were scarcely present (β_1-integrin and dystrophin accounted for 21 and 4% respectively of fraction 23F2) (Figure 4). These properties allowed us to consider fraction

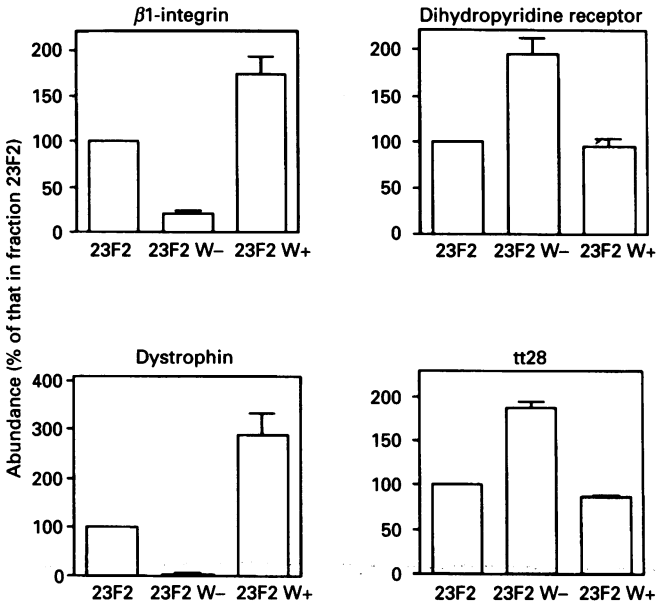

Figure 4 Separation of T-tubules and sarcolemmal membranes from cell-surface fraction 23F2

The abundance of β_1-integrin, dystrophin, dihydropyridine receptors and tt28 protein was assayed in 23F2 fractions before and after agglutination with WGA lectin. 23F2 fractions were incubated with WGA and subsequently the agglutinated vesicles (23F2 W^+) were separated from the non-agglutinated fractions (23F2 W^-). The distribution of surface markers was studied by immunoblot analysis. Equal amounts of membrane protein (1–3 μg) from the different fractions were loaded on the gels. Autoradiograms were subjected to scanning densitometry. The results (means ± S.E.M.) of six experiments are expressed as the percentage of levels detected in fractions 23F2.

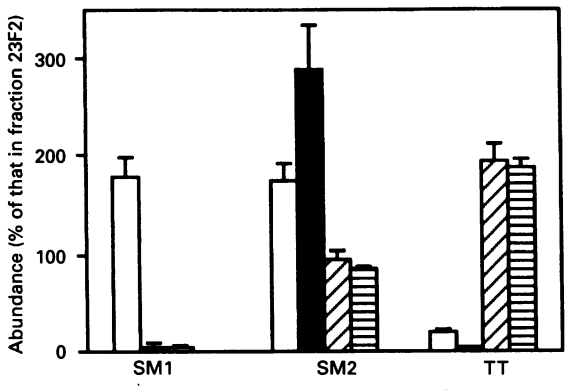

Figure 5 Characterization of sarcolemmal fractions and T-tubules

The abundance of β_1-integrin (□), dystrophin (■), dihydropyridine receptors (▨) and tt28 protein (▤) was assayed in 23F1 and 23F2 fractions before and after agglutination with WGA lectin. Fractions 23F1 and 23F2 were incubated with WGA and the agglutinated vesicles were then separated from the non-agglutinated fractions (see the legend to Figure 6). Fraction 23F1 W^+ was defined as sarcolemmal fraction 1 (SM1), 23F2 W^+ as sarcolemmal fraction 2 (SM2) and 23F2 W^- as the T-tubule fraction (TT). The distribution of surface markers was studied by immunoblot analysis. Equal amounts of membrane protein (1–3 μg) from the different fractions were loaded on the gels. Autoradiograms were subjected to scanning densitometry. The results (means ± S.E.M.) of six experiments are expressed as the percentage of levels detected in fraction 23F2.

23F2 W^- as a highly purified T-tubule fraction (TT in Figure 5). Further characterization revealed the presence of GLUT4-type glucose carrier in both membrane fraction SM2 and T-tubules (results not shown).

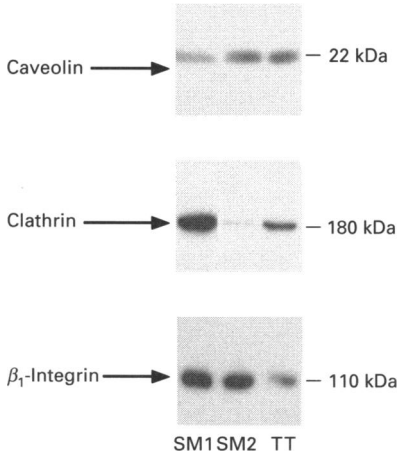

Figure 6 Distribution of caveolin and clathrin heavy chain in sarcolemmal fractions and T-tubules

The abundance of caveolin and clathrin heavy chain was assayed in SM1, SM2 and T-tubule fraction (TT) (see details in legend to Figure 7). The abundance of β_1-integrin was determined in parallel. The distribution of caveolin, clathrin heavy chain and β_1-integrin was studied by immunoblot analysis using specific antibodies. Equal amounts of membrane protein (1–6 μg) from the different fractions were loaded on the gels. Autoradiograms were subjected to scanning densitometry.

In summary, the processing of sequential low- and high-speed homogenates from rat skeletal muscle followed by WGA treatment permits the purification of : (a) SM1, which is characterized by a high abundance of β_1-integrin and is dystrophin-free, (b) SM2, which highly enriched in both β_1-integrin and dystrophin, and (c) a T-tubule fraction, which is highly enriched in dihydropyridine receptors and tt28 and essentially devoid of membranes of sarcolemmal origin (Figure 5).

Distribution of caveolin and clathrin in cell-surface membranes of the muscle fibre

On the basis of their role in cell-sorting processes, we next studied the distribution of proteins associated with clathrin-coated and non-clathrin-coated membrane invaginations. To this end, we determined the abundance of caveolin, a protein found in caveolae or caveolin-rich domains [39] and clathrin heavy chain, a component of the clathrin structure [40], in the three different cell-surface membrane populations SM1, SM2 and T-tubules.

Caveolin migrated as a 22 kDa protein on SDS/PAGE and was detected in all three cell-surface fractions examined. SM2 and T-tubules contained more caveolin than SM1 (Figure 6). These data suggest that caveolae or caveolin-rich domains are located in all the cell-surface membrane domains obtained.

We also examined the abundance of the clathrin heavy chain in parallel. It was essentially restricted to the membrane fractions enriched in cell-surface components (i.e. fractions 23F1 and 23F2) (results not shown). In addition, clathrin heavy chain was very abundant in the SM1 fraction but was also present, although to a lesser extent, in T-tubule membrane fractions (Figure 6). Only trace amounts were detected in the SM2 fraction.

Immunoisolation of T-tubule vesicles

Prior experiments depicted in Figure 4 allowed the isolation of a membrane fraction highly enriched in T-tubules that contained

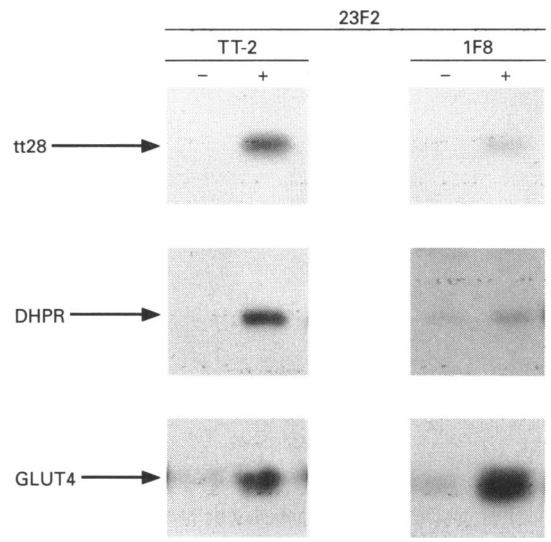

Figure 7 Immunoadsorption of T-tubule fractions with immobilized antibody TT-2 and 1F8

Membrane vesicles 23F2 obtained from skeletal muscle were incubated with or without immobilized antibodies 1F8 or TT-2. After incubation, the adsorbed fractions were electrophoresed and immunoblotted to determine the abundance of tt28 protein, dihydropyridine receptors (DHPR) and GLUT4. Autoradiographs were subjected to scanning densitometry. Representative autoradiograms obtained after various times of exposure are shown. Results (means ± S.E.M. of five to seven experiments) after vesicle immunoisolation are expressed as a percentage of specific immunoadsorption. In experiments using TT-2 antibody, the percentage of specific immunoadsorption was 19.0 ± 1.2, 16.2 ± 6.4 and 17.1 ± 3.2% for tt28, GLUT4 and dihydropyridine receptors respectively. In experiments using 1F8 antibody, the percentage of specific immunoadsorption was 5.1 ± 1.0, 36.4 ± 5.5 and 0.6 ± 0.4% for tt28, GLUT4 and dihydropyridine receptors respectively.

abundant levels of dihydropyridine receptors, tt28 protein and GLUT4. In order to provide additional proof of the co-localization of these proteins in T-tubules, we performed immunoisolation of T-tubule vesicles (Figure 7). In an initial set of experiments, 23F2 fractions were subjected to immunoisolation by using monoclonal antibody TT-2, which specifically recognizes tt28 protein [24]. Under our experimental conditions, TT-2 specifically immunoadsorbed 19% of the tt28 present in fraction 23F2 (legend to Figure 7). Vesicles immunoisolated with antibody TT-2 also contained dihydropyridine receptors and GLUT4 (Figure 7). The percentage of GLUT 4 and dihydropyridine receptors specifically immunoadsorbed ranged between 13 and 22% of the total present in fraction 23F2 (legend to Figure 7). Co-localization of GLUT4, tt28 and dihydropyridine receptors in vesicles derived from T-tubules is in accord with the data obtained by WGA agglutination of fraction 23F2 (Figure 4).

23F2 vesicles were also immunoadsorbed with monoclonal antibody 1F8 which is specific for GLUT4 [26]. Nearly 40% of all GLUT4 found in fraction 23F2 was specifically immunoadsorbed with 1F8 (Figure 7). Under these conditions, tt28 and dihydropyridine receptors were also detected in the immunoadsorbed fraction (Figure 7). The percentage of tt28 and dihydropyridine receptors specifically immunoadsorbed ranged between 0.6 and 5% of total protein present in fraction 23F2 (legend to Figure 7). No tt28 or dihydropyridine receptors were found in the pellet resulting from non-specific immunoadsorption (Figure 7). The different proportions of proteins found after immunoadsorption with TT-2 and 1F8 antibodies suggests the existence of different domains in T-tubules.

DISCUSSION

In this study, a protocol of subcellular fractionation of membranes from rat skeletal muscle was set up that involved sequential low- and high-speed Polytron homogenization of rat skeletal muscle. The rationale for a sequential homogenization step was based on reports that low-speed homogenization allows the isolation of sarcolemmal membranes with little contamination from sarcoplasmic reticulum [41], whereas high-speed homogenization is routinely performed when the objective is to isolate substantial amounts of T-tubule membranes [21,42]. Characterization analysis indicated that the profile of cell-surface membrane markers was different from that of sarcoplasmic Ca^{2+}-ATPase or intracellular GLUT4-containing vesicles. On this basis, we conclude that the protocol of subcellular fractionation established here is suitable for the isolation of different cell membranes from the muscle fibre, cell-surface membranes as well as intracellular membranes. Isolation of distinct membrane domains from a single preparation of skeletal muscle might be useful in obtaining a precise understanding of cellular processes that affect different membrane populations.

The processing of sequential low- and high-speed homogenates followed by treatment with WGA has revealed the existence of subcompartments within the sarcolemma of muscle fibre; these two subcompartments, named SM1 and SM2 in the present study, share some proteins, such as caveolin and β_1-integrin, whereas other proteins, such as dystrophin and clathrin heavy chain, are exclusively located in a single domain. Thus dystrophin is found in SM2 but not in SM1 and clathrin heavy chain is very abundant in SM1 but is absent from SM2. A complementary distribution of vinculin and dystrophin has recently been reported in two distinct sarcolemmal domains of smooth muscle [43]. Our results suggest that fractions SM1 and SM2 mainly contain membranes from the muscle fibre and not from other cell types present in skeletal muscle, for instance, those derived from perineural sheaths or endothelial cells. This is based on several pieces of evidence: (a) all cell-surface membrane fractions obtained contained markers that are specific for the muscle fibre, such as dystrophin, GLUT4, protein tt28 and dihydropyridine receptors, and the major cell type in skeletal muscle is the muscle fibre, and (b) no membrane proteins known to be expressed in peripheral nervous tissue, such as synaptophysin, were detected in any of the membrane fractions obtained (results not shown). [44].

The demonstration of a heterogeneous distribution of dystrophin in sarcolemma (present in SM2 and absent from SM1) is consistent with recent morphological observations reported in human, guinea-pig, rat and mouse skeletal muscle [45–48]. On the basis of the preferential distribution of dystrophin in sarcolemmal domains overlying the I bands [37,38], we suggest that SM2 represents a fraction enriched in the sarcolemmal domains located in the vicinity of T-tubules approaching the surface. Thus we conclude that our protocol for the purification of cell-surface membrane fractions from a single muscle preparation is suitable for use in the study of the distribution of membrane proteins in the muscle fibre.

Analysis of subcellular fractions revealed that most GLUT4 is intracellular under basal fasting conditions. Nearly 18% was in cell-surface membranes and the rest was in intracellular membranes that did not co-localize with sarcoplasmic reticulum. In addition, our results indicate that, under basal conditions, GLUT4 is present in T-tubules. This conclusion was based on the following findings: (a) GLUT4 was detected in highly purified T-tubule membranes free from sarcolemmal components, and (b) there was co-localization of GLUT4 and T-tubule markers

(protein tt28 and dihydropyridine receptors) after immunoadsorption of T-tubule vesicles with either monoclonal antibody TT-2 directed against tt28 or monoclonal antibody 1F8 directed against GLUT4. These observations confirm previous reports of GLUT4 protein in T-tubules from human and rat skeletal muscle [20,49,50].

T-tubule-vesicle immunoisolation assays showed that utilization of immobilized antibody TT-2 raised against tt28 resulted in immunoadsorption of vesicles which contain abundant protein tt28, dihydropyridine receptors and GLUT4. In contrast, utilization of antibody 1F8 against GLUT4 led to immunoisolation of vesicles enriched in GLUT4 but showing a low content of tt28 and dihydropyridine receptors. In addition, insulin administration *in vivo* increased the amount of GLUT4 immunoadsorbed after immunoisolation of T-tubule vesicles (P. Muñoz, unpublished work). These results indicate the existence of two distinct domains in muscle-fibre T-tubules: one population of T-tubule vesicles shows co-localization of GLUT4 and T-tubule markers such as tt28 and dihydropyridine receptors, whereas the other shows a high abundance of GLUT4 but a low content of tt28 and dihydropyridine receptors. A thorough evaluation of different domains in the T-tubule of the muscle fibre requires vesicle immunoisolation analysis using immobilized antibodies against proteins specifically localized in the T-tubule.

Our results suggest the existence of distinct membrane domains in both the sarcolemma and T-tubules of muscle fibre. In the sarcolemma, complementary distributions of dystrophin and clathrin heavy chain define two distinct sarcolemmal domains (SM1 and SM2); in contrast, some proteins are distributed in a similar manner in these two subcompartments. For proteins specific to T-tubule or specific to selective domains of the sarcolemma, a specific intracellular transport pathway for each selective cell-surface domain seems likely. In contrast, targeting of proteins resident in both the T-tubules and the sarcolemma or in different domains of the sarcolemma may be accomplished by one of two mechanisms: either such proteins are specifically routed to either system or they are incorporated into one compartment and then redistributed throughout the continuous membrane systems. In any case, our results suggest a complete set of exocytic and endocytic processes controlling the fine distribution of membrane proteins in the cell surface of the muscle fibre.

In this regard, it is interesting that clathrin heavy chain was essentially detected in SM1 and T-tubule fractions only, whereas SM2 was devoid of this protein. These observations suggest that clathrin-coated invaginations are not randomly distributed throughout the cell surface of the muscle fibre but are only found in T-tubules and selective domains of the sarcolemma characterized by the absence of dystrophin. A corollary is that endocytosis mediated via clathrin-coated pits is restricted to selective domains of the cell surface in the muscle fibre. Furthermore, the presence of clathrin in T-tubules provides a mechanistic explanation for the recycling of proteins in these cell-surface invaginations.

In contrast with the distribution of clathrin, we found that caveolin is abundantly present in distinct sarcolemmal membrane fractions as well as in T-tubules. Caveolin, also named VIP21 [51,52], which was the first protein to be identified as a component of the caveolar coat [53], is believed to play an important role in sorting processes, transmembrane signalling and molecular transport across membranes [39]. Our results indicate that it is located in all cell-surface membrane fractions analysed. This supports the view that sarcolemma is an active compartment with regard to the formation and maintenance of caveolae or caveolin-rich domains. The presence of caveolin in T-tubule membranes supports previous morphological observations that T-tubules frequently terminate in caveolae [54,55] and the finding that dihydropyridine receptors are also localized in caveolae in rabbit skeletal muscle [56].

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