Insulin-Induced Redistribution of GLUT4 Glucose Carriers in the Muscle Fiber

In Search of GLUT4 Trafficking Pathways

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Insulin rapidly stimulates glucose transport in muscle fiber. This process controls the utilization of glucose in skeletal muscle, and it is deficient in various insulin-resistant states, such as non-insulin-dependent diabetes mellitus. The effect of insulin on muscle glucose transport is mainly due to the recruitment of GLUT4 glucose carriers to the cell surface of the muscle fiber. There is increasing evidence that the recruitment of GLUT4 carriers triggered by insulin affects selective domains of sarcolemma and transverse tubules. In contrast, GLUT1 is located mainly in sarcolemma and is absent in transverse tubules, and insulin does not alter its cellular distribution in muscle fiber. The differential distribution of GLUT1 and GLUT4 in the cell surface raises new questions regarding the precise endocytic and exocytic pathways that are functional in the muscle fiber. The current view of insulin-induced GLUT4 translocation is based mainly on studies performed in adipocytes. These studies have proposed the existence of intracellular compartments of GLUT4 that respond to insulin in a highly homogeneous manner. However, studies performed in skeletal muscle have identified insulin-sensitive as well as insulin-insensitive intracellular GLUT4-containing membranes. These data open a new perspective on the dynamics of intracellular GLUT4 compartments in insulin-sensitive cells. *Diabetes* 45 (Suppl. 1):S70–S81, 1996

Skeletal muscle accounts for nearly 40% of body mass and is the main tissue involved in the insulin-induced stimulation of glucose uptake. Several studies using the euglycemic-hyperinsulinemic clamp have shown that, at circulating levels of insulin in the upper physiological range, most of the infused glucose is taken up by skeletal muscle and converted mainly into glycogen (1–3). Evidence for the role of muscle glucose uptake in overall glucose homeostasis also comes from studies in transgenic mice overexpressing GLUT1 in skeletal muscle. Thus overexpression of GLUT1 in transgenic mice causes increased glucose uptake in muscle associated with an enhanced expression of GLUT1, which leads to low plasma glucose concentrations and an increased glucose disappearance rate after a glucose tolerance test (4).

Insulin treatment, exercise, or electrical stimulation rapidly stimulates the rate at which glucose is taken up across the cell surface (5–9). Kinetic analysis of the effects of insulin or contraction on muscle glucose transport indicates that they are a consequence of an enhancement in V_{max} values (6,10). The effects of insulin and exercise on glucose uptake or glucose transport in muscle are fully additive (10–13) or synergistically additive (14–17), which has been interpreted as being due to the triggering of different and interacting mechanisms. A complicating factor when dealing with skeletal muscle is the considerable heterogeneity of insulin action among muscles of different fiber compositions (18–20). Red muscle exhibits three- to fourfold higher sensitivity and maximal responsiveness to insulin with respect to 2-deoxyglucose uptake (18,19), glycogen synthesis (20), and amino acid uptake (12) than white muscle. Some responsibility for this effect might be attributable to intrinsic differences of insulin receptor kinase activity in red and white muscle (21) or to a consequence of or differences in the expression level of glucose transporters (22–24).

The rate of muscle glucose transport is thought to be a rate-limiting step in the pathway of glucose utilization in skeletal muscle. Several observations support this view: *1*) intracellular free glucose does not accumulate in normal skeletal muscle and muscle rendered insulin resistant in streptozotocin-induced diabetic rats, regardless of glucose or insulin concentration (25,26); *2*) under normoglycemic conditions, both in the absence of insulin and at submaximal insulin concentrations, glucose clearance is constant in the perfused rat hindlimb (27); and *3*) overexpression of GLUT1 in skeletal muscle from transgenic mice leads to a 10-fold increase in muscle glycogen content and a 2-fold increase in muscle lactate, with no increase in muscle glucose-6-phosphate concentrations (28).

Another aspect of considerable interest is the evidence available indicating that patients with non-insulin-dependent diabetes mellitus (NIDDM) show deficient insulin-induced glucose transport in skeletal muscle (29,30). Furthermore, nuclear magnetic resonance measurements have demonstrated that deficient glucose transport or glucose phosphorylation is responsible for the lower rate of muscle glycogen synthesis found in NIDDM patients during a hyperglycemic-hyperinsulinemic clamp (31). All these observations clearly strengthen the importance of understanding the biochemical

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NIDDM, non-insulin-dependent diabetes mellitus.

pathways triggered by insulin that lead to the stimulation of glucose transport in skeletal muscle.

DISTRIBUTION OF GLUCOSE CARRIERS IN THE MUSCLE FIBER UNDER BASAL CONDITIONS

To maintain a rate of glucose transport across the membrane that can be stimulated by a variety of factors, such as insulin or the degree and intensity of exercise, the skeletal muscle expresses the glucose transporter isoforms GLUT4 and GLUT1 (32–37). The GLUT3 glucose transporter was cloned initially from an expression library obtained from human fetal muscle (38). However, Northern blot analysis performed with RNA preparations from fetal, neonatal, or adult rat skeletal muscle does not support the expression of GLUT3 mRNA in this tissue (A. Castelló, A.Z, unpublished observations).

Besides GLUT4 and GLUT1, there is some evidence that skeletal muscle of humans but not of rats expresses GLUT5 (39,40). Based on the induction of fructose transport activity obtained in *Xenopus* oocytes after injection of human GLUT5 cRNA (41), it has been hypothesized that GLUT5 accounts for fructose uptake by muscle. However, the precise functional role of GLUT5 in muscle deserves further study.

GLUT4 is quantitatively the most important glucose carrier expressed in skeletal muscle from adult rats, and GLUT1 accounts for only 5–10% of total glucose carriers (37), which is consistent with data previously reported in isolated rat adipocytes (42). However, the relative abundance of GLUT1 and GLUT4 in muscle depends on the developmental stage (43). Thus, during fetal and early postnatal life, GLUT1 is the predominant glucose transporter isotype expressed in skeletal muscle, and the onset of GLUT4 induction occurs during late fetal life (43). During the perinatal period, a transition occurs in muscle by which GLUT4 expression rises and GLUT1 is repressed (43). During this period, the expression of GLUT1 and GLUT4 glucose transporter in muscle is controlled by innervation-dependent basal contractile activity (44) and by thyroid hormones (45).

GLUT4 expression is restricted to the muscle fiber and the cardiomyocyte in skeletal muscle and heart, respectively (Fig. 1). In an initial report, GLUT4 was immunodetected both in muscle cells and in endothelial cells from cryosections of rat skeletal muscle and rat heart (46). These studies were performed using monoclonal antibody 1F8, which has been shown to detect GLUT4 protein specifically in immunoblot and in immunoprecipitation assays (33,42). Immunofluorescence labeling of GLUT4 in muscle and in endothelial cells with antibody 1F8 was shown to be specific, and it was blocked by preabsorption of 1F8 with a peptide corresponding to the COOH terminus of GLUT4. Later experiments using different polyclonal antibodies against the COOH terminus of GLUT4 did not show any labeling of GLUT4 in endothelial cells (47) (Fig. 1). Further studies performed using endothelial cells obtained from a variety of sources such as bovine aorta, human umbilical vein, and rat heart microvessels did not detect GLUT4 protein or mRNA (F. Viñals, A.Z., unpublished observations). Therefore, whether antibody 1F8 reacts, under the conditions used for immunofluorescence or immunogold analysis, in a specific manner with a membrane protein resident in endothelial cells that is not GLUT4 remains to be determined.

The currently available evidence indicates that, under basal conditions, GLUT1 and GLUT4 show a differential localization in the muscle fiber. Results obtained from subcellular fractionation studies indicate that GLUT1 carriers are located mainly in a fraction enriched in plasma membrane markers such as 5′-nucleotidase, Mg^{2+}-ATPase, or Na^{+}-K^{+}-ATPase activities (34,35,37), whereas GLUT4 carriers are mostly abundant in membrane fractions containing intracellular markers such as galactosyltransferase (34,35, 48). In our laboratory, we have also examined the distribution of GLUT4 and GLUT1 glucose carriers in different fractions obtained in skeletal muscle from overnight-fasted rats by using a new protocol of subcellular fractionation (49). This protocol involves the sequential low- and high-speed polytron homogenization of rat skeletal muscle. The rationale for a sequential homogenization step was based on different reports that low-speed homogenization allows the isolation of sarcolemmal membranes with little contamination with sarcoplasmic reticulum (50), whereas high-speed homogenization is performed routinely to isolate substantial numbers of transverse-tubule (T-tubule) membranes (51,52). After homogenization, membrane fractions were washed with high concentrations of KCl to separate membranes from myofibrils and subsequently loaded with Ca^{2+} to differentially enhance the density of membranes derived from the sarcoplasmic reticulum. Sucrose gradient centrifugation of membranes derived from the low-speed homogenization step (F1 fraction) and membranes derived from the high-speed homogenization step (F2 fraction) allowed the isolation of different membrane populations (Fig. 2). The membrane fractions showing the maximal enrichment in cell surface components were found in the lightest fractions of the gradient (fractions 23F1 and 23F2, the membrane fractions recovered on top of the 23% sucrose layer of the sucrose density gradient; Fig. 2) (49). In parallel, GLUT1 was maximally abundant in the lightest fractions of the sucrose density gradients (Fig. 2) under basal conditions, which is consistent with the view that GLUT1 carriers are localized mainly in the cell surface. In contrast, our data confirmed that GLUT4 was localized mainly intracellularly. Thus the greatest abundance of GLUT4 was found in denser membrane fractions of the sucrose density gradients (the maximal abundance was found in membrane fractions 26F1 and 26F2, and high levels were also found in fractions 29F1 and 29F2; Fig. 2). Our subcellular fractionation data indicate that under basal conditions (i.e., low endogenous circulating insulin concentrations), GLUT4 and GLUT1 glucose carriers present in the cell surface fractions account for 18 and 55%, respectively, of the total amount present when all membrane fractions obtained are pooled. The distribution of GLUT4 in cell surface membranes is in good agreement with immunoelectron microscopy data.

Morphological studies have also provided evidence that under basal conditions GLUT4 is mainly intracellular and that GLUT1 is found in the cell surface in muscle fiber. Immunofluorescence analysis using antibodies against GLUT4 labeled both the cell periphery and the interior in transverse cryosections of rat skeletal muscle (37). In contrast, antibodies against GLUT1 specifically labeled the cell periphery but not the interior in rat skeletal muscle, as found in immunofluorescence assays (37). Furthermore, immunoelectron microscopy of human vastus lateralis (53), rat soleus muscle (54–56), and rat extensor digitorum longus (Fig. 3) has shown that GLUT4 labeling is associated mostly

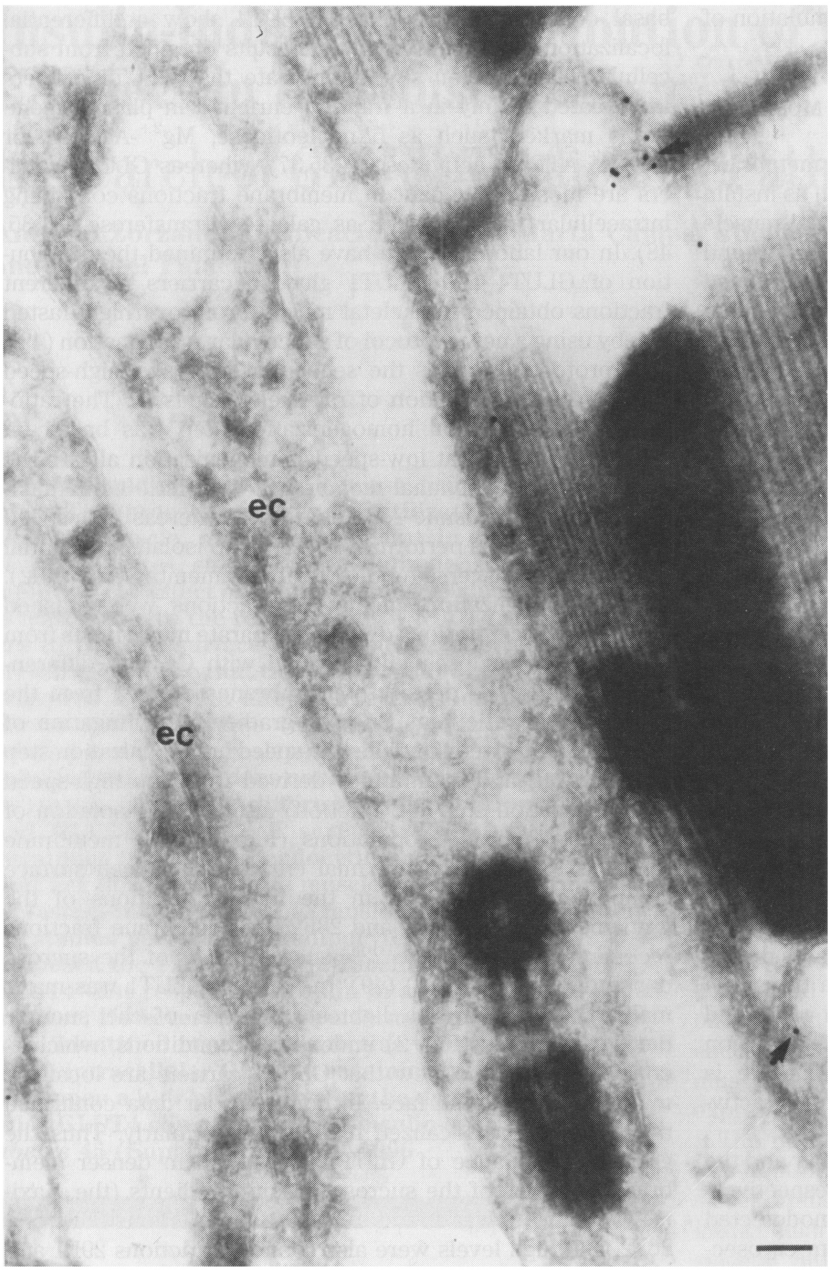

FIG. 1. Cryosections of rat heart labeled for GLUT4. Rat heart cryo-ultrasections were incubated with polyclonal antibody OSCRX against GLUT4 (1:400 dilution). Antibody OSCRX was produced in rabbit after immunization with a peptide corresponding to the final 15 amino acids of the COOH terminus of GLUT4 (121). The localization of GLUT4 was revealed with protein A–colloidal gold (15 nm gold). ec, endothelial cells; arrow, labeling associated with T tubule. Scale bar: 100 nm.

with intracellular membranous vesicular structures (Fig. 3). These tubulovesicular elements essentially occur in four locations in extensor digitorum longus muscle: *1*) perinuclear tubulovesicular elements near the Golgi apparatus (Fig. 3*A*), *2*) between myofibrils (Fig. 3*E*), *3*) near the sarcolemma (Fig. 3*B*), and *4*) near the T-tubule membranes (Fig. 3*C*). Quantification of GLUT4 labeling in rat extensor digitorum longus muscle indicated that 88% of total specific labeling was found in tubulovesicular elements inside the muscle fiber.

The muscle fiber is characterized by the existence of distinct domains in the cell surface, i.e., the sarcolemma and the T tubules. The sarcolemma covers the muscle fiber and contains membrane proteins involved in the transport or recognition of different solutes (57–59). Furthermore, there is evidence that some membrane proteins might be distributed rather heterogeneously throughout the sarcolemma. Thus morphological observations performed in human, guinea pig, rat, and mouse skeletal muscle (60–63) have reported the preferential distribution of dystrophin in sarcolemmal domains overlying the I bands. In addition, a complementary distribution of vinculin and dystrophin has been reported recently in two distinct sarcolemmal domains in smooth muscle (64). Structurally continuous with the surface sarcolemma and invaginating into the muscle fiber is the T-tubule membrane system. In addition to providing access to the interior of the muscle cell for extracellular fluid, 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 calcium release by the sarcoplasmic reticulum. 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, β-adrenergic receptors, or G-proteins (59,65–67), as well as a distinct set of membrane

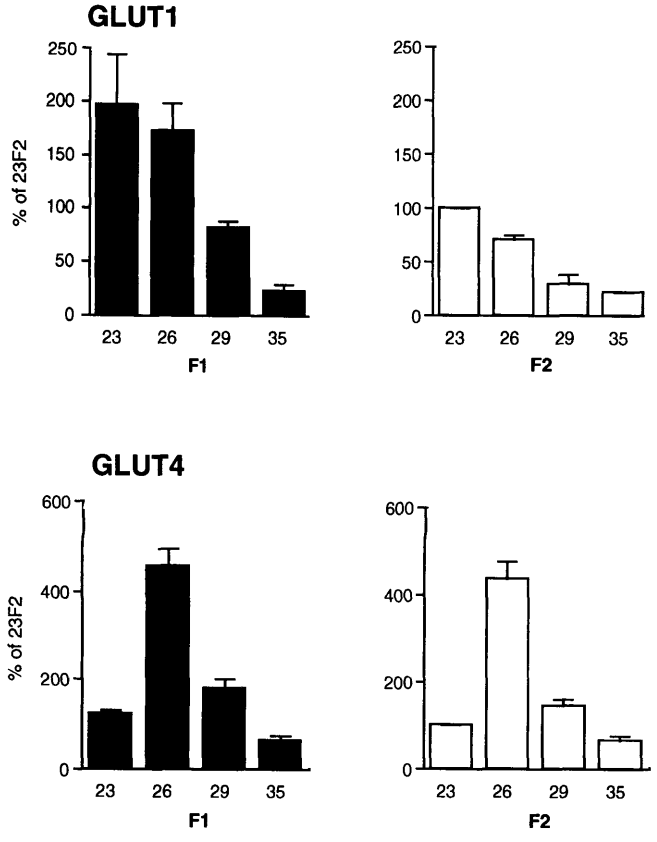

FIG. 2. Distribution of GLUT1 and GLUT4 in different membrane populations resulting after subcellular fractionation of rat skeletal muscle membranes. The abundance of GLUT1 and GLUT4 was assayed in fractions obtained after sucrose gradient centrifugation of membranes obtained after low-speed homogenization (F1 fraction) and high-speed homogenization (F2 fraction) of rat skeletal muscle. Fraction 23 (from F1 or F2) was collected on top of 23% sucrose, fraction 26 from the interphase 23–26% sucrose, fraction 29 from the interphase 26–29%, and fraction 35 from the interphase 29–35%. The distribution of GLUT1 and GLUT4 was studied by immunoblot analysis using specific antibodies. Equal amounts of membrane proteins were laid on gels (1–3 μg). Autoradiograms were subjected to scanning densitometry. The results are means ± SE of four to six experiments. Data were expressed as percentage of values found in fraction 23F2.

proteins, some of which are involved in excitation-contraction coupling, such as the dihydropyridine receptor (67,68).

In our laboratory, we have isolated three different cell surface membrane fractions (sarcolemmal fraction 1, sarcolemmal fraction 2, and T-tubule membranes) from a single preparation of rat skeletal muscle (49) by using agglutination in wheat germ agglutinin, as described previously (69,70). The biochemical characterization of these cell surface membrane fractions is shown in Table 1. Sarcolemmal fraction 1 shows a high enrichment in β_1-integrin and lacks dystrophin, two different markers of sarcolemma (71–73). In contrast, sarcolemmal fraction 2 shows a high content of β_1-integrin and dystrophin. Sarcolemmal membrane fractions 1 and 2 also differ in their abundance of clathrin heavy chain; clathrin is highly abundant in sarcolemmal fraction 1, whereas it is absent in sarcolemmal fraction 2. T tubules show a high abundance of the specific T-tubule markers tt28 protein and dihydropyridine receptors (68,74) and lack sarcolemmal markers (Table 1). Our results demonstrate further the existence of subcompartments within sarcolemma of the muscle fiber. In addition, our experimental protocol for the subcellular fractionation of membranes represents a suitable tool to determine the distribution of membrane proteins in different cell surface domains in the muscle fiber.

Based on all the information mentioned above, the question of whether GLUT4 and GLUT1 glucose carriers are distributed homogeneously in all cell surface components in the muscle fiber is relevant. Initial studies performed by Burdett et al. (58) showed that membrane preparations enriched in T tubules contained three- to fivefold more glucose carriers (as measured by cytochalasin B binding) than membrane preparations enriched in sarcolemma. Later subcellular fractionation studies have provided evidence that both sarcolemmal and T-tubule membrane preparations contain GLUT4 carriers (75,76), which are more abundant per unit of membrane protein in T-tubule membrane preparations (76) (Table 1). Quantitative analysis of immunoelectron microscopy observations performed in rat extensor digitorum longus muscle also supports this view. Thus, under basal conditions, 8% of total GLUT4 labeling was found in T tubules, and 3% of total labeling was detected in sarcolemma (Fig. 3). Additional proof for the presence of GLUT4 in T tubules in muscle under basal conditions was obtained by immunoisolation of T-tubule vesicles by using monoclonal antibody TT2, which specifically recognizes tt28 protein (49). Vesicles immunoisolated with antibody TT2 contained protein markers of T tubules, such as tt28 and dihydropyridine receptors, as well as GLUT4.

Interestingly, the insulin receptor is also distributed heterogeneously in the cell surface of the muscle fiber. Under basal conditions, insulin receptors are found mainly in the cell surface (76). Furthermore, insulin receptors are detected both in T tubules and in selective domains of sarcolemma, as shown by subcellular fractionation analysis and by immunoisolation of T tubules (Fig. 4 and Table 1).

Subcellular distribution of GLUT1 shows a different pattern than GLUT4. Both immunofluorescence analysis and subcellular fractionation data (37,75,76) show that GLUT1 carriers in the cell surface are found in the sarcolemma and absent from T tubules under fasted conditions. In addition, GLUT1 glucose transporters are distributed heterogeneously throughout the sarcolemma (76), and GLUT1 abundance is greater in sarcolemmal fractions rich in clathrin and free from dystrophin (Table 1).

In summary, the data currently available indicate that, under basal conditions, GLUT4 and GLUT1 are targeted to distinct domains of the cell surface; also, whereas GLUT1 is mainly found in sarcolemma and is excluded from the T tubule, surface GLUT4 is more abundant in T tubules than in sarcolemma. This suggests the existence of separate exocytic machineries involved in the arrival of GLUT1 and GLUT4 carriers at the cell surface of the muscle fiber (Fig. 4). For GLUT1, an intracellular transport pathway delivering membrane proteins specifically to sarcolemma is likely to be required. In contrast, targeting of GLUT4 carriers, resident in both the T tubules and sarcolemma, may be accomplished by one of two mechanisms: GLUT4 carriers either are routed specifically to either cell surface compartment or are incorporated into one compartment and then redistributed throughout the continuous membrane systems. In any case, these results suggest a complex set of exocytic and endocytic processes controlling the distribution of glucose carriers in different cell surface domains of the muscle fiber. It is estimated that *1*) GLUT4 is 10-fold more abundant than GLUT1 in skeletal muscle, *2*) 3 and 8% of all cellular GLUT4

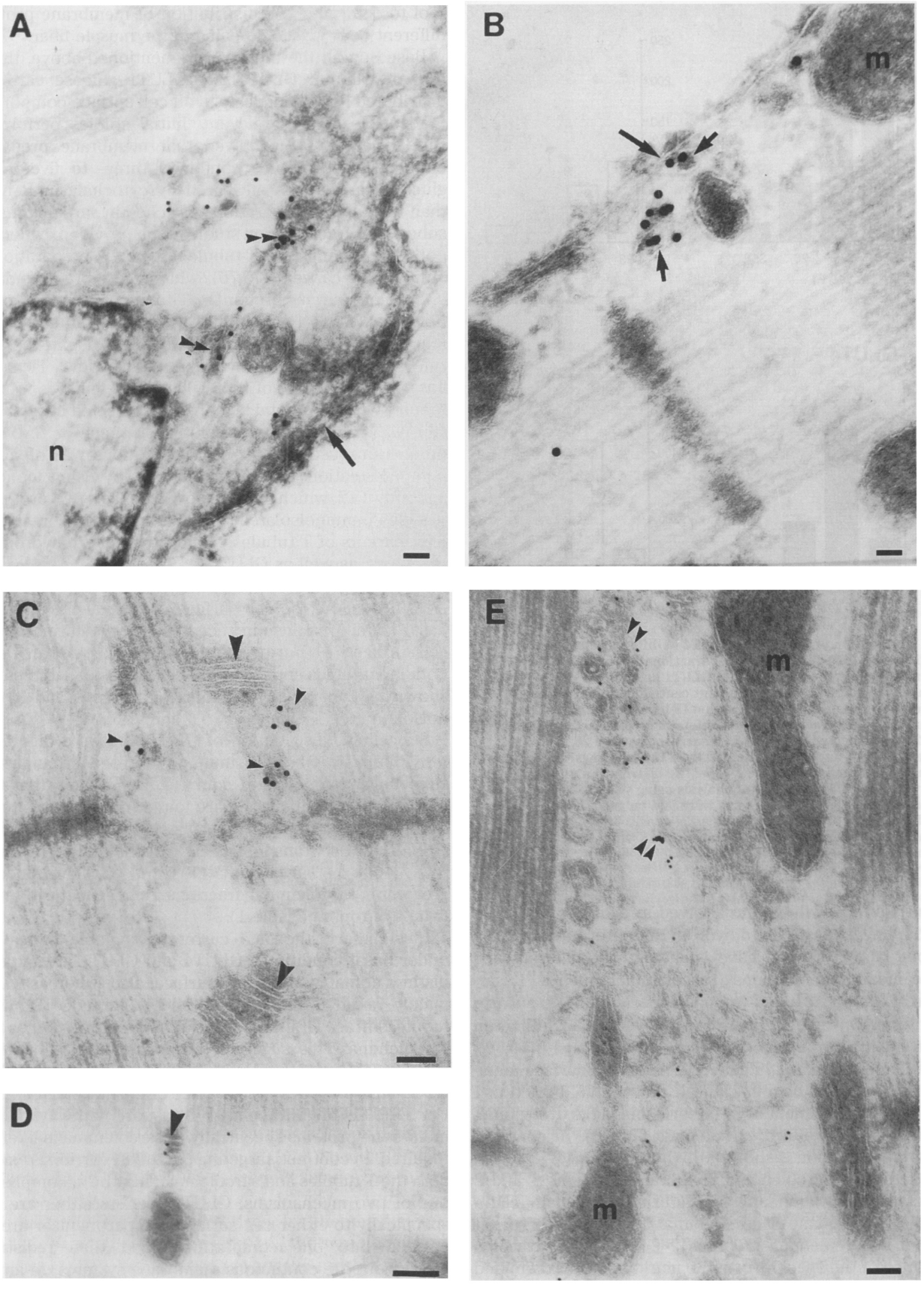

FIG. 3. *Cryosections of nonstimulated rat extensor digitorum longus muscle labeled for GLUT4. Extensor digitorum longus muscle cryo-ultrasections obtained from overnight-fasted rats were incubated with polyclonal antibody OSCRX against GLUT4 and protein A–colloidal gold (10, 15, and 20 nm gold). A:* strong labeling of vesicular elements (double arrowheads) near the nucleus (n). Arrow, sarcolemma. Scale bar: 100 nm (15 nm gold). *B:* GLUT4 is found in subsarcolemmal vesicles (short arrows) close to the sarcolemma (long arrow). m, mitochondria. Scale bar: 100 nm (20 nm gold). *C:* labeling of intracellular vesicles (small arrowheads) near the T tubule (large arrowheads). Scale bar: 100 nm (15 nm gold). *D:* labeling of the T tubule (arrowhead). Scale bar: 200 nm (10 nm gold). *E:* labeling of intracellular vesicles found between myofibrils (contiguous arrowheads). Scale bar: 100 nm (10 nm gold).

TABLE 1
Distribution of membrane proteins in different sarcolemmal membrane fractions and in transverse tubules from rat skeletal muscle under fasted conditions

	Sarcolemmal membrane 1	Sarcolemmal membrane 2	T tubules
β_1-Integrin	++	++	−
Dystrophin	−	++	−
Clathrin	++	−	+
Caveolin	++	++	++
GLUT1	++	+	−
GLUT4	+	+	++
Insulin receptor	−	+	++
tt28	−	+	++
Dihydropyridine receptors	−	+	++

β_1-Integrin, dystrophin, clathrin heavy chain, caveolin, GLUT1, GLUT4, insulin receptors, tt28, and dihydropyridine receptors were assayed in sarcolemmal membrane fractions 1 and 2 and T-tubule membranes obtained from rat skeletal muscle (49,76). − and + indicate the absence and presence of a certain protein in a particular fraction, respectively; ++ indicates that levels of a certain protein were the highest in a particular fraction compared with the other fractions.

is present in sarcolemma and T tubules, respectively, under basal fasted conditions, and *3*) 60% of all GLUT1 is in the sarcolemma; based on this, we postulate that under nonstimulated conditions most of the glucose uptake through sarcolemma is catalyzed by GLUT1 and that GLUT4 is responsible for glucose taken up through T tubules.

INSULIN STIMULATES GLUT4 TRANSLOCATION IN THE MUSCLE FIBER

The initial observation that insulin stimulated the translocation of glucose carriers from an intracellular locus to the plasma membrane in isolated rat adipocytes (77,78) was followed by different attempts to demonstrate an analogous mechanism in muscle. Early studies performed in rat diaphragm showed that insulin caused a twofold increase in glucose transporter number in plasma membrane fractions and a concomitant decrease in intracellular glucose transporters (79,80). Further studies of subcellular fractionation in skeletal muscle also showed that insulin administration led to an increased glucose transporter number in plasma membrane and a decrease in intracellular glucose transporter content, in agreement with insulin's promoting the recruitment of glucose carriers to the cell surface (50,81). Muscle contraction has also been shown to increase the level of glucose transporters in plasma membrane in rat skeletal muscle (82–87).

The development of tools to immunodetect specific glucose carrier isotypes has facilitated the study of the translocation of glucose carriers induced by insulin or exercise. Immunocytochemical studies (53–56), subcellular fractionation (34,35,37,48,76), and photolabeling assays (88,89) indicate that GLUT4 moves from an intracellular locus to the cell surface of the muscle fiber in response to insulin administration. In contrast, GLUT1 is not recruited in response to insulin in rat skeletal muscle (34,35). Exercise also recruits GLUT4 to the cell surface (91); however, indirect evidence suggests that exercise or muscle contraction depletes intracellular GLUT4 pools different from the compartments recruited by insulin (90). Whether insulin or muscle contraction also modifies the intrinsic activity of GLUT4 carriers remains unknown.

Regarding the cell surface involved in insulin-induced GLUT4 recruitment, it has been shown that GLUT4 is recruited to the sarcolemma (54–56). Figure 5 shows immunoelectron microscopy data of GLUT4 protein in nonstimulated and insulin-treated extensor digitorum longus muscles. In vivo insulin injection enhanced the labeling of sarcolemma in muscle fiber (Fig. 5; specific labeling of the sarcolemma was 3 and 12% of total specific labeling in nonstimulated and insulin-stimulated muscle fibers, respectively). However, under these conditions the total specific labeling per unit area did not differ with the control group. Subcellular fractionation analysis performed in our laboratory has revealed that only selective domains of sarcolemma are affected by GLUT4 recruitment in response to insulin (76). Thus GLUT4 is recruited by insulin to sarcolemmal membrane fractions highly enriched in dystrophin but not to sarcolemmal membrane fractions not containing dystrophin and highly enriched in clathrin (76).

Insulin also promotes the translocation of GLUT4 to the T tubules of the muscle fiber. This conclusion is based on the following observations: *1*) GLUT4 abundance increases in membrane preparations highly enriched in T tubules and devoid of sarcolemmal vesicles from insulin-treated groups (37,76), *2*) immunoisolation of T-tubule vesicles with antibody TT2 specific for tt28 (a T-tubule marker) leads to greater recovery of GLUT4 in the immunoprecipitates from the insulin-treated group than in those from the control group (76), *3*) immunoelectron microscopy studies have detected the translocation of GLUT4 to T tubules in human vastus lateralis muscle (53), and *4*) the amount of ATB-[2-^{3}H]BMPA photolabeled in T tubules from intact incubated

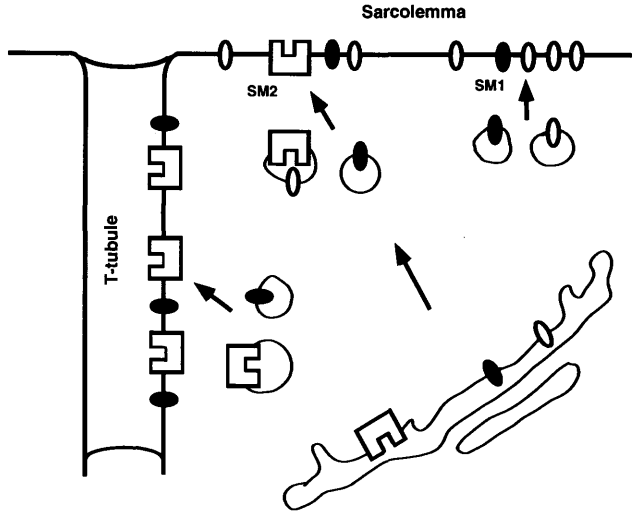

FIG. 4. Scheme of the hypothetical distribution of glucose carriers and insulin receptors in the cell surface of the muscle fiber under basal fasted conditions. ◊, GLUT1; ♦, GLUT4; ▭, insulin receptors. The scheme shows the distribution of glucose carriers and insulin receptors in T tubules and in distinct domains of the sarcolemma. SM1 and SM2, sarcolemmal membranes 1 and 2, respectively (see Table 1 for detailed characterization). Under basal conditions, most GLUT1 and insulin receptors are found in the cell surface and GLUT4 is mainly intracellular. The aim of this scheme is to show the distribution of glucose carriers and insulin receptors in the different domains of the cell surface in the muscle fiber; it is not meant to show the relative proportions of proteins in the cell surface vs. in the intracellular compartment. The intracellular proteins are indicated only to suggest possible (and as yet hypothetical) exocytic pathways involved in the arrival of glucose carriers and insulin receptors at the cell surface.

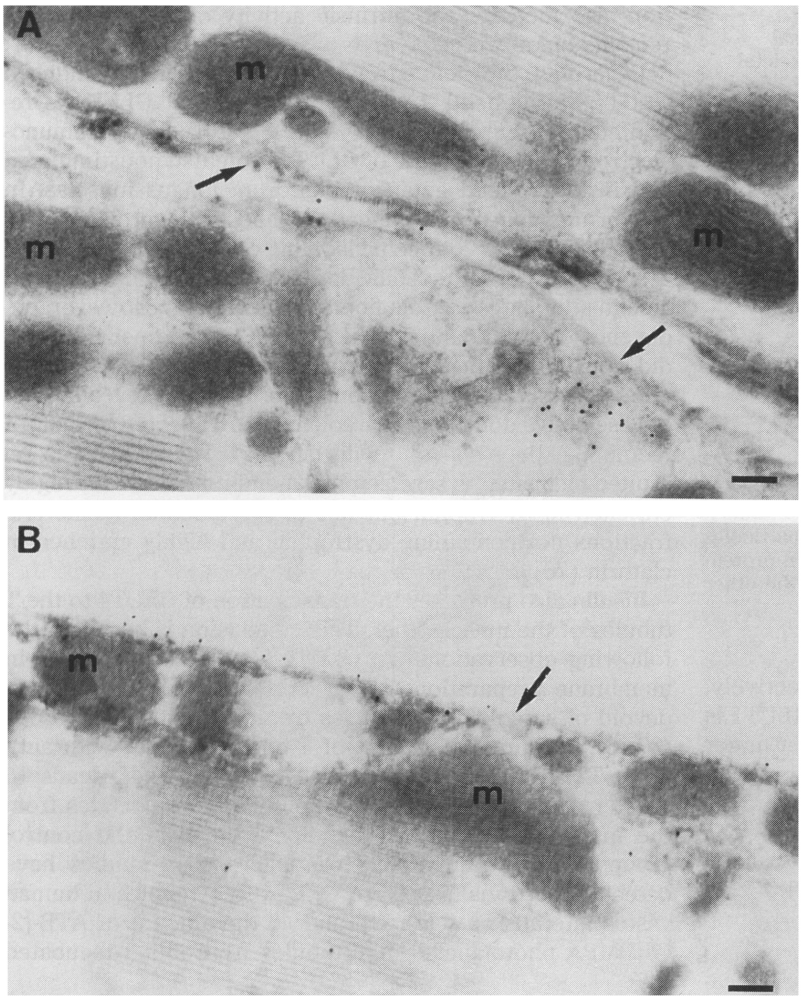

FIG. 5. Cryosections of extensor digitorum longus muscle from nonstimulated and insulin-treated rats labeled for GLUT4. Extensor digitorum longus muscle cryo-ultrasections obtained from overnight-fasted rats were incubated with polyclonal antibody OSCRX against GLUT4 and protein A–colloidal gold (10 nm gold). m, mitochondria. Scale bars: 200 nm. *A:* under nonstimulated conditions, most peripheral labeling is found in subsarcolemmal vesicles and not in the sarcolemma (arrows). *B:* after stimulation with insulin, substantial labeling is detected along the sarcolemma (arrows).

soleus muscles was increased in response to insulin, as assessed by autoradiography (92).

In all, current evidence indicates that, in response to acute insulin treatment, GLUT4 is translocated to selective domains of sarcolemma and also to T tubules of the muscle fiber (Fig. 6). Based on the fact that insulin recruits GLUT4 to a dystrophin-enriched sarcolemmal fraction and on the selective distribution of dystrophin in sarcolemma (63), we suggest that this is a costameric sarcolemmal domain (Fig. 6). A similar process of insulin-induced GLUT4 recruitment affecting both sarcolemma and T tubules has also been described in rat cardiomyocytes (93). A corollary that requires experimental validation is that insulin enhances the uptake of glucose through both sarcolemma and T tubules in the muscle fiber.

The finding that GLUT4 is translocated to different domains of the cell surface in response to insulin poses the question of where insulin action is initiated in the muscle fiber. In this regard, we have found that insulin-induced GLUT4 recruitment occurs in those membrane fractions containing insulin receptors. These observations indicate that both sarcolemma and T tubules are target domains for the initiation of insulin action in the muscle fiber, which suggests that insulin action in T tubules and sarcolemma is triggered and regulated in an autonomous manner.

The insulin-induced GLUT4 translocation to T tubules is certain to have an important role in muscle physiology. The muscle fiber is a thick structure, with an average diameter of 50 μm; therefore the uptake of glucose through the T tubules after stimulation by insulin might be a way to enhance the efficiency of channeling glucose toward its metabolic fates. An aspect that requires experimental analysis is whether, as postulated by some authors, the T tubule is too narrow to allow efficient diffusion of metabolites. The proposal of a direct arrival of exogenous glucose at the muscle fiber via the T tubules fits with several previous observations. Glycolytic enzymes show a heterogeneous distribution in the muscle fiber, and they are found preferentially in a cytosolic compartment surrounding the I band, i.e., close to T tubules (94–96). Glycolytic enzymes such as aldolase and glyceraldehyde-3-phosphate dehydrogenase are tightly bound to isolated triads from skeletal muscle (97–99). Mitochondria display two distinct locations in the muscle fiber: peripherally close to the sarcolemma and packed in between the myofibrils, preferentially in a transverse plane between the triadic system and the Z disk (100), i.e., close to the T tubules. Furthermore, insulin increases the binding of hexokinase to mitochondria in muscle (101,102), which may increase the efficiency of oxidative phosphorylation (103). In summary, the arrival of glucose through the T tubule would channel glucose directly to hexokinase bound to the outer mitochondrial membrane, near the T tubules, and then to the rest of the glycolytic enzymes, also located in the vicinity of T tubules.

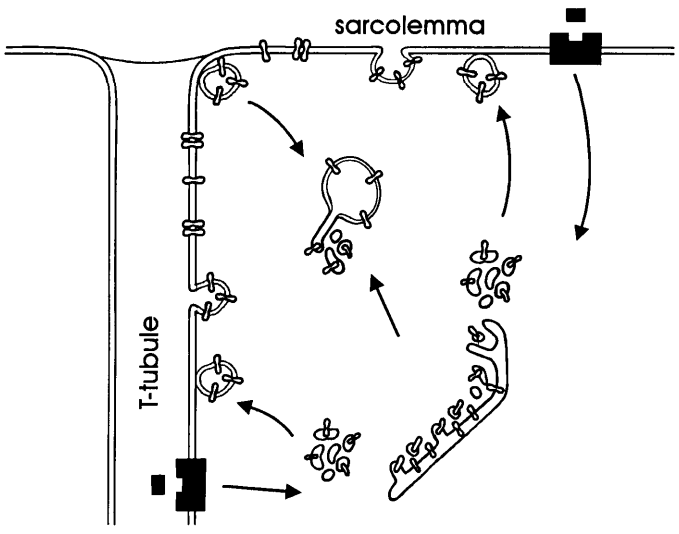

FIG. 6. Scheme of the hypothetical translocation of GLUT4 in the muscle fiber in response to insulin. ▯, GLUT4; ▬, insulin receptors; ■, insulin. Insulin binds to insulin receptors present both in sarcolemma and in T tubules (1), which triggers insulin signaling (2). As a consequence, insulin promotes GLUT4 translocation from intracellular compartments to sarcolemma and T tubules (3). Under both basal and insulin-stimulated conditions, GLUT4 is subjected to internalization from cell surface membranes (4).

INTRACELLULAR GLUT4 COMPARTMENTS IN THE MUSCLE FIBER

As mentioned previously, immunoelectron microscopy of human vastus lateralis (53), rat soleus (54–56), and rat extensor digitorum longus muscles (Fig. 3) has shown that GLUT4 labeling is mostly associated with intracellular membranous vesicular structures (Fig. 3). In extensor digitorum longus muscle, these tubulovesicular elements are found in a perinuclear location close to the Golgi apparatus and in the proximity of the sarcolemma or the T tubules (Fig. 3). At present, we have no information regarding the possible functional role of the different GLUT4 pools identified by morphological means.

Subcellular fractionation analysis has identified an intracellular pool of GLUT4 vesicles obtained from rat skeletal muscle that becomes depleted of GLUT4 after insulin treatment (34,35,48,90). However, although some subcellular fractionation protocols obtain an intracellular GLUT4 pool that is depleted in response to exercise (91), other studies obtain an intracellular GLUT4 pool that is depleted by insulin but not by acute exercise (35,90). These observations have allowed researchers to postulate that exercise-sensitive GLUT4 transporters do not originate necessarily from the insulin-sensitive intracellular membrane compartment. Consequently, this concept points to the existence of various intracellular GLUT4 pools in the muscle fiber, showing a different response to signals. In any event, any future working hypothesis dealing with the cell biology of the distinct intracellular GLUT4 pools responding to various signals should explain the fact that muscle contraction and exercise are fully or synergistically additive activators of glucose transport.

Linked to this issue, we have found in our laboratory that, concomitantly with the GLUT4 recruitment affecting sarcolemma and T tubules, insulin treatment causes a marked decrease in the content of GLUT4 in some intracellular vesicles (intracellular membrane fractions originating from the low-speed homogenization step previously defined, which represent 28% of the total intracellular GLUT4 pool) but not in other fractions (intracellular membrane fractions originating from the high-speed homogenization step, which represent 72% of the total intracellular GLUT4 pool) obtained from subcellular fractionation of rat skeletal muscle (49,76). These findings again suggest the existence of several pools of intracellular GLUT4 carriers in skeletal muscle. The nature of the intracellular insulin-insensitive and -sensitive GLUT4 pool, its distribution in the muscle fiber, and its biochemical composition remain to be determined. Another urgent question is the physiological role of the insulin-insensitive GLUT4 pool. This all requires deeper knowledge of the molecular architecture of these different membranous vesicular populations.

Our data have also revealed that in response to insulin, ~12% of the intracellular GLUT4 was redistributed to the cell surface. These results may agree with previous findings from Rodnick et al. (55) indicating that the combined stimulation with insulin and acute intense exercise causes a redistribution of 40% of intracellular GLUT4 in skeletal muscle. These observations substantiate the existence of a large intracellular reservoir of GLUT4 compared with the requirements of insulin action in skeletal muscle. Furthermore, this large excess intracellular GLUT4 pool might elucidate the additive or synergistic effect of insulin and exercise on glucose transport substantiated in skeletal muscle (10–17,22).

One logical way to define the biological role of different intracellular GLUT4 pools is to characterize the proteins associated with GLUT4 in the same vesicles. Recent studies have identified some of the proteins that colocalize in GLUT4-containing vesicles from rat adipocytes. These include phosphatidylinositol-4-kinase (104), vesicle-associated membrane proteins (105), secretory carrier membrane proteins/GTV3 (106,107), gp160 (108–110), and some low–molecular weight GTP-binding proteins including rab4 (111, 112). The precise role of these proteins is currently unknown. There is very little information regarding the biochemical composition of GLUT4 vesicles from skeletal muscle. Vesicle-associated membrane protein and gp160 proteins are also expressed in skeletal muscle (109,113), and it is important to determine whether they colocalize with GLUT4 in the same vesicles. Recent studies have found that insulin-induced GLUT4 recruitment to the cell surface of the muscle fiber was concomitant with translocation of GTP-binding proteins (114). The functional relationship between GLUT4 and GTP-binding proteins remains unestablished.

In our laboratory, we have initiated the characterization of the intracellular GLUT4 compartment from rat skeletal muscle. To this end, in some initial experiments we explored the abundance of GLUT4 as well as different membrane proteins in membrane fractions obtained by subcellular fractionation of rat skeletal muscle. From the different proteins analyzed, we detected a number of membrane proteins to be highly abundant in intracellular membrane fractions enriched in GLUT4 (Fig. 7). A protein subjected to study was caveolin, the caveola coat protein, which is believed to play an important role in sorting processes, transmembrane signaling, or molecular transport across membranes (115). Caveolin was present in all cell surface domains of the muscle fiber (Table 1), and it was highly abundant in intracellular membrane fractions, following a pattern similar to GLUT4 (Fig. 7). Furthermore, intracellular membrane fractions that con-

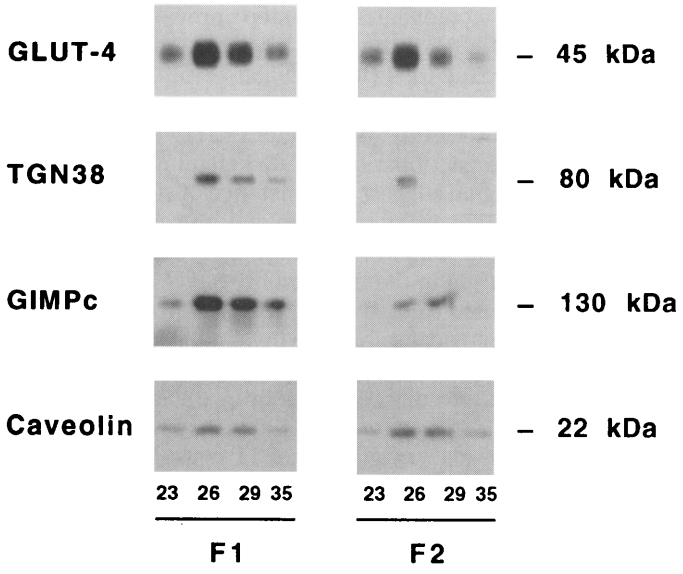

FIG. 7. Distribution of GLUT4, TGN38, GIMPc, and caveolin in membrane populations resulting after subcellular fractionation of rat skeletal muscle membranes. The abundance of GLUT4, TGN38/GIMP$_{T-1}$, GIMPc, and caveolin was assayed in fractions obtained after sucrose gradient centrifugation of membranes obtained after low-speed homogenization (F1 fraction) and high-speed homogenization (F2 fraction) of rat skeletal muscle. Fraction 23 (from F1 or F2) was collected on top of 23% sucrose, fraction 26 from the interphase 23–26% sucrose, fraction 29 from the interphase 26–29%, and fraction 35 from the interphase 29–35%. The distribution of the different membrane proteins was studied by immunoblot analysis by using specific antibodies. Equal amounts of membrane proteins (1–3 μg) from the different fractions were laid on gels. Representative autoradiograms are shown. Times of exposure for one particular protein were the same for F1 and F2 fractions.

tain high levels of GLUT4 were also enriched in GIMPc and TGN 38/GIMP$_{T-1}$, known markers of the *cis*- (116) and *trans*-Golgi networks (116–118), respectively (Fig. 7).

Based on these data, we performed further studies to determine the possible colocalization of GLUT4 with caveolin and TGN38. To this end, intracellular GLUT4-enriched membrane fractions were subjected to immunoisolation by using monoclonal antibody 1F8, which specifically recognizes GLUT4. Under our experimental conditions, 1F8 specifically immunoadsorbed 70% of the GLUT4 present in fractions (Fig. 8). Vesicles immunoisolated with antibody 1F8 only contained trace amounts of caveolin and TGN38, supporting the view that most of the GLUT4 does not colocalize with caveolin or TGN38. It appears, therefore, that the *trans*-Golgi network is not a major site of the intracellular GLUT4 pool within the muscle fiber. In fact, a similar lack of colocalization between GLUT4 and TGN38 has been shown in 3T3-L1 adipocytes (119). Our data are in agreement with recent data obtained by Hundal et al. (120) indicating that the disruption of the *trans*-Golgi network does not interfere with insulin-like growth factor I–dependent glucose transport in L6 muscle cells in culture. In addition, our data indicate that the recycling of GLUT4 and caveolin occurs via independent pathways.

Currently available information regarding insulin-induced GLUT4 translocation in muscle fiber can be summarized according to the following model (Fig. 6). Under fasted conditions, most GLUT4 is intracellular in the vicinity of Golgi apparatus, T tubules, and sarcolemma. It is unlikely that a substantial amount of GLUT4 is in the *trans*-Golgi network. Insulin causes the translocation of GLUT4 to T tubules and to selective domains of the sarcolemma, and the cell surface domains affected by GLUT4 recruitment also contain insulin receptors. Redistribution of intracellular GLUT4 only affects a small percentage of total 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 two 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 in the muscle fiber.

FUTURE PROSPECTS

The detailed understanding of the mechanisms by which insulin stimulates glucose transport in skeletal muscle is a major task that will contribute to the development of therapeutic strategies for patients with insulin resistance or NIDDM. In the last few years, we have learned a great deal about the cell biology of GLUT4 in adipose cells, but we are just beginning to understand how GLUT4 is targeted in the muscle fiber. GLUT4 glucose carriers seem to be translocated to both sarcolemma and T tubules; however, we do not know yet the relative importance of each particular cell surface domain in regard to the effect of insulin in the muscle fiber. The nature and biochemical characteristics of the intracellular GLUT4 compartments that are recruited by insulin also remain unknown. Several major technical problems must be solved before these questions can be answered completely: *1*) the subcellular fractionation of skeletal muscle is fraught with difficulties and, as a result, the membrane yields are low compared with those obtained in other insulin-sensitive cells or tissues; and *2*) muscle cells in culture currently available do not form T tubules, so they represent cell models that are hardly comparable with the muscle fiber. In this regard, the achievement of better subcellular fractionation protocols, the development of muscle cells in culture more comparable with muscle fibers, the

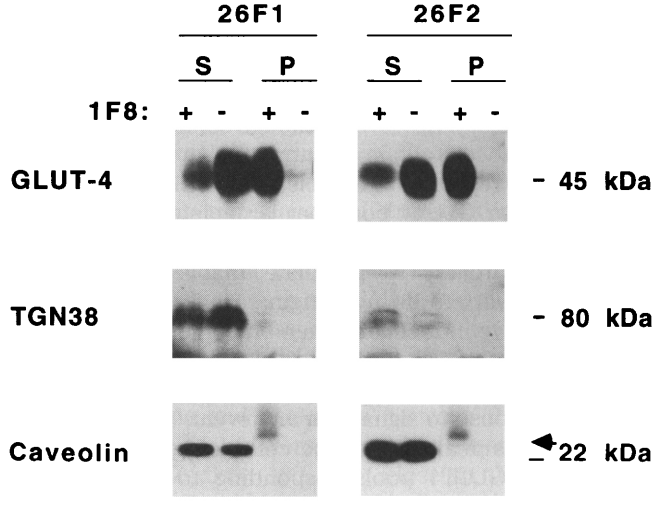

FIG. 8. Immunoadsorption of intracellular GLUT4-containing vesicles with immobilized antibody 1F8. Membrane vesicles of intracellular origin (membrane fractions 26F1 and 26F2) obtained from rat skeletal muscle were incubated with (+) or without (–) immobilized antibody 1F8. After the incubation, the adsorbed fractions (P) and supernatants (S) were electrophoresed and immunoblotted to determine the abundance of GLUT4, TGN38, and caveolin. Autoradiographs were subjected to scanning densitometry. Representative autoradiograms obtained after various times of exposure are shown. The arrow indicates the presence of immunoglobulin G light chain.

improved understanding of the cell biology of the muscle fiber, and the production of transgenic animals defective in proteins critical for GLUT4 recruitment are appropriate lines of research to be pursued in the future.

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