Skeletal muscle accounts for up to 40–60% of the human body mass and is the major site of glucose disposal, thereby regulating whole body glucose homeostasis. Insulin resistance in skeletal muscle has been proposed to be the primary defect in type 2 diabetes (10, 11, 16). Insulin-stimulated blood glucose disposal is principally mediated through glucose transport across the muscle cell surface (i.e., glucose entry into muscle fibers), and this step can be a rate-limiting step for glucose clearance (5). Glucose crosses the plasma membranes and enters skeletal muscle through the glucose transporters by way of facilitated transport (5).

Fully differentiated skeletal muscle fibers have a unique and complex cellular architecture developed when mononucleated myoblasts during fetal and neonatal state fuse into G1-arrested polynucleated myotubes (Fig. 1). As myotubes mature, formation of characteristic cross- striations coincides with the buildup of protein-rich regions of the contractile apparatus, eventually dispersing the nuclei to the periphery (Fig. 1). A membrane channel system responsible for propagating neural signals consisting of deep invaginations of the cell membrane surface (the sarcolemma) is also formed (Fig. 2). These structures, named the transverse tubules (T-tubules) comprise the majority of the mature muscle fiber membrane surface, which has been estimated to be one to four times the surface area of the plasma membrane proper (8, 40, 51, 61). The T-tubules have a well-established role in distribution of the nerve-induced membrane depolarization during excitation-contraction coupling, and their significant contribution to glucose transport is beginning to be appreciated (36, 37, 61). Together, these structural features make the long (up to several centimeters in length) and polynucleated muscle fibers vastly different from the other major insulin-responsive cell type, the adipocyte. The adipocyte is, in contrast, a mononucleated cell of much smaller size, with only one surface membrane and a cytosol filled with lipids and not contractile proteins.

Glucose uptake into skeletal muscle is principally regulated by two transporter proteins, GLUT1 and GLUT4. GLUT4 has been shown to be the primary insulin-responsive glucose transporter in skeletal muscle. Transcript levels of GLUT4 have been reported to be approximately 400-fold higher than GLUT1 in human skeletal muscle, and it appears that a significant portion of the GLUT1 present in human muscle tissue may reside outside of mature muscle fibers primarily in the vascular endothelial cells, although detectable amounts were present in the sarcolemma as well (59). GLUT12 was recently shown to parallel some of the characteristics of GLUT4 in clonal cell cultures and human skeletal muscle (58); however, relatively little is known about the regulation or importance of GLUT12 in muscle and as such is outside the scope of this review. GLUT1 resides primarily in the sarcolemma and not intracellular depots or T-tubules (59, 61), thereby mediating the basal glucose uptake into the muscle fiber. In contrast, only a small percentage of total GLUT4 protein is located on the cell surface, since in the absence of sufficient stimuli GLUT4 is localized to one or several not yet fully characterized vesicular storage compartments within the muscle fiber. A fraction of GLUT4 has been proposed to recycle between internal vesicular stores and the membrane surfaces in the absence of stimuli, and recent studies suggest that this basal recycling fraction of GLUT4 in the T-tubules significantly contributes to

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basal glucose uptake (36, 37). Upon stimulation with insulin (i.e., in the postprandial phase) or muscle contraction (i.e., during exercise) or other stimuli (i.e., hypoxia or metabolic stress), GLUT4 transporters are translocated from internal vesicular stores to the sarcolemma and the T-tubules (37, 51, 61), and the cell surface density of GLUT4 coincides with the rate of glucose transport (22, 49, 51, 61). The signaling cascades leading to insulin- and muscle contraction-mediated GLUT4 translocation have been shown to be independent and additive with respect to GLUT4 translocation and glucose uptake (20, 49, 51, 61). Given that the T-tubules are such an abundant membrane surface in muscle fibers, they have also been reported to be the major site of insulin signaling, GLUT4 translocation, and glucose uptake in skeletal muscle (35–37, 61). During insulin-resistant states, insulin-induced GLUT4 translocation and glucose uptake into muscle are compromised (19, 65), whereas the contraction-induced responses are often intact (27, 65), highlighting the need for elucidating the similarities and divergence in these signaling cascades and the intracellular traffic mechanisms responsible for altering the magnitude of cell surface GLUT4.

However, despite decades of intense research on GLUT4, many molecular and cellular details of GLUT4 translocation dynamics and regulation still remain obscure. Although many details of the insulin signaling cascade are known, consequent regulation of GLUT4's intracellular traffic still lack significant layers of detail. In addition, our understanding of GLUT4 regulation during muscle contraction is poorly developed compared with insulin. The understanding of GLUT4 traffic dynamics in skeletal muscle is hindered because of technical limitations rooted in the inherent complexity of this tissue. The large, long muscle fibers containing thousands of nuclei and large amounts of contractile proteins are embedded together with tendon attachments and capillaries in a complex three-
dimensional structure (Fig. 2A). This structure has made muscle cell sorting, isolation, staining, and subcellular fractionation very complex and challenging. Sample preparation underlies many of the technical barriers that complicate GLUT4 detection in skeletal muscle. For example, it is thought that in vivo or in situ assessments may be hindered by capillary access and the endothelial barrier, whereas additional complications may arise during in vitro assessments. These barriers include, but are not limited to, the facia (epimysium), a layer of connective tissue that envelopes the muscle, the perimysium, inward projections of the facia that segregate bundles of fibers (fascicles), the endomysium, another layer of connective tissue that surrounds each muscle fiber within the fascicles (Fig. 2A). Finally, as described previously, the sarcolemma of a muscle fiber (although continuous) contains deep invaginations, T-tubules, which may be inaccessible to certain compounds or proteins, depending on the preparation used for assessment (Fig. 2B). Further adding complexity are abundant specialized membrane compartments like the calcium releasing sarcoplasmic reticulum and mitochondria, together with the high content of contractile proteins in the myofibrils making up the majority of the muscle cell interior (Fig. 2B). In addition, the traditional cellular compartments like the Golgi complex and the endosomal compartment are fragmented and distributed throughout the muscle cell in unconnected elements (50, 51) (Fig. 2B). To date, many of the investigations on GLUT4 regulation in skeletal muscle have been indirect and have had a limited spatial-temporal resolution. For example, surgically excised in vitro muscle preparations and perfused hindlimb preparations have been used to estimate GLUT4 translocation based on GLUT4 content in different subcellular fractionation membrane pools or by indirect measurements like glucose transport (42, 49). However, these types of techniques have a limited time resolution, since only a few time points are practically available (i.e., basal and 10 or 30 min poststimulation). Furthermore, compartmentalized signaling or traffic events within the muscle cell are not available with indirect measurements such as glucose transport or when the cellular architecture is homogenized and fractionated into a few membrane pools (with potential cross-contamination) as in subcellular fractionation studies. Indeed, a major hindrance of fractionation studies is the inability to confidently discern between GLUT4 that is inserted into the plasma membrane or T-tubules (and available to transport glucose) and transporters that reside in intracellular depots and are not available to transport glucose.

Thus, in order to improve spatial resolution, studies have employed electron (EM) or fluorescence microscopy of immuno-stained GLUT4 in skeletal muscle fibers or muscle cross sections, which have provided both quantitative and spatial information regarding the location of GLUT4 vesicles during basal and insulin- and contraction-stimulated conditions (51, 61). Despite the improvement in the spatial information, the time resolution of these studies is still very limited, with a typical experiment measuring basal conditions and one time point after a given stimulation. Furthermore, an extensive series of invasive technical steps is required to prepare samples for either EM- or immunofluorescence-based imaging. These methods may introduce potential detection artifacts, such as high background (or nonspecific signal), which can lead to misleading results. In addition, when used in skeletal muscle, these techniques have not yielded dynamic (or kinetic) information regarding how GLUT4 traffics before, during, and after stimulation (i.e., endocytosis/exocytosis), through which compartments GLUT4 vesicles move during translocation, and how the traffic is regulated to and from various muscle membrane surfaces. Furthermore, small changes in GLUT4 localization might be masked (or introduced) due to staining variation (or simply variation in GLUT4 expression/localization between muscle fibers) when one compares different muscles or muscle fiber types stained for GLUT4, which is alleviated if the same GLUT4 vesicles are monitored before, during, and after stimulation. To overcome these resolution obstacles in mature muscle, research has turned to GLUT4 translocation studies in cultured cell systems.

Cell Systems Used to Study GLUT4 Translocation with High Spatial-Temporal Resolution In Vitro

In order to avoid the limited spatial-temporal resolution of GLUT4 traffic studies in mature muscle, in vitro cultured muscle cells have been widely used to measure GLUT4 translocation kinetics. In vitro GLUT4 translocation models, hypothesized to recapitulate some of the features of mature muscle include mononuclear myoblasts and polynucleated myotubes (Fig. 1). Depending on the source of the muscle cells, which can range from fetal or adult myoblasts isolated from rodent or human biopsies or myoblasts from immortal cell lines (i.e., C2C12 or L6 myoblasts), the formation of myotubes reaches different levels of myogenic differentiation and maturity (Fig. 1). For example, rat L6 myotubes develop into polynuclear fiber-like structures that express endogenous GLUT4, but these myotubes do not have cross-striations or T-tubules (52). In contrast, C2C12-derived myotubes have low endogenous expression of GLUT4 but do (although only to a minor extent) develop T-tubules and cross-striations and display some features of muscle contraction given an appropriate stimulus (7, 45, 46). Despite that, clonal cell lines are genetically stable; the phenotype of the differentiated myotube depends heavily on the protocol used to induce differentiation (45). Alternatively, other studies probing GLUT4 traffic necessitated the use of mononuclear systems such as L6 myoblasts or non-muscle cell types such as 3T3-L1 fibroblasts and 3T3-L1 adipocytes (14, 17, 47). These cell models are imperative in assessing various aspects of GLUT4 cell biology but are further removed from mature skeletal muscle.

One of the problems inherent in myotube- and myoblast-based cell systems is that they do not recapitulate the features of fully differentiated mature muscle (Figs. 1 and 2). This is due to many factors, including the inability of in vitro systems to adequately simulate the in vivo environment, including neural innervation, presence of endogenous hormonal factors, constant passive tension and mechanical load (i.e., stretch), and cyclical contraction-relaxation cycles. In addition, endogenous GLUT4 expression is lower in many cell culture systems compared with mature muscle [i.e., a 5- to 10-fold lower GLUT4 expression in L6 myotubes (24)] and in general have been reported to have a lower insulin-stimulated glucose transport compared with skeletal muscle (2-fold in L6 myotubes compared with 3- to 10-fold in muscles in vivo) depending on the methodology used (30, 31, 49). Furthermore, most myotube cultures lack mature T-tubules either completely [L6 muscle
cells (52) or only partially [C2C12 muscle cells (7)], a membrane surface that in mature muscle has been suggested to be the predominant site of GLUT4-mediated glucose uptake (37, 51, 61). It is noteworthy that isolating mature muscle fibers for subsequent in vitro culture may not circumvent this problem, since these fibers will rapidly (within a few days) dedifferentiate and lose many of the features of mature muscle due to the lack of innervation (18).

Despite these shortcomings, muscle cell culture models such as rat L6 myoblasts and myotubes stably (or transiently) expressing GLUT4 with an exofacial or fluorescent protein tag have been valuable in characterizing GLUT4 traffic events in high spatial-temporal resolution using single cell fluorescence microscopy and cell population-based assays (3, 23, 25). These systems allow for assessment of GLUT4 exocytosis, endocytosis, and defects in various steps of GLUT4 traffic to be analyzed in intact cells with a time resolution of choice. The malleability of in vitro systems is one of their biggest advantages, given the relative ease of GLUT4 measurements and availability of genetic and/or chemical manipulation of the cells. The green fluorescent protein (GFP) tag (on GLUT4) is useful in direct monitoring of intracellular GLUT4 traffic, but given the resolution limit of light it cannot discern GLUT4-GFP inserted into the plasma membrane from GLUT4-GFP residing in close juxtaposition (<200 nm) to the surface membrane. Thus, muscle cells stably or transiently expressing GLUT4 with an exofacial tag [myc or hemagglutinin (HA)] have been used to determine levels of cells surface GLUT4. Myotubes have been fixed at specific time points and labeled with a surface membrane impermeant anti-myc antibody in order to measure GLUT4-myc (with or without GFP) inserted into the plasma membrane by detecting the exofacial loop containing the myc epitope (44). The tags have been combined, including GLUT4-myc-GFP, which allows simultaneously the detection of the GLUT4-GFP and the fraction of GLUT4-myc actually inserted into the membranes (15, 44).

The myotube-based studies have provided important dynamic information regarding the regulation of GLUT4 traffic. For example “pulse-chase” experiments have shown that insulin-mediated GLUT4-myc translocation to the cell surface was due to an increase in exocytosis of GLUT4-containing vesicles and not a reduction in endocytosis (38). Despite the important and extensive work on GLUT4 translocation or traffic performed in the various in vitro models, these models differ significantly in cellular architecture and differentiation level from mature muscle. Thus, in vitro findings have to be validated in vivo. Fortunately, recent technical advances in labeling and imaging techniques have provided opportunities for GLUT4 labeling and dynamic analysis in mature muscle fibers.

Investigating GLUT4 Translocation in Mature Muscle

To date, several different approaches have been used to study GLUT4 translocation in mature muscle fibers. One approach has been to analyze the localization and movement of GLUT4-GFP directly in situ in anesthetized animals (33, 35–37) (Fig. 3). Another approach has been to isolate mature muscle fibers or cardiomyocytes from transgenic mice overexpressing tagged (myc, HA, and/or GFP) versions of GLUT4 and subsequently perform analysis in vitro (15, 54). Alternatively, the use of various generations of membrane impermeable affinity photolabels that bind to endogenous glucose transporters have also provided important information about GLUT4 traffic in mature muscle (26) (Fig. 3).

In Situ Expression of Tagged GLUT4: Transient Expression

Transient overexpression of GLUT4-GFP in mouse or rat muscle fibers has been achieved by using either intramuscular injection of naked plasmid vectors alone (33) or combined with electroporation (35) or gene gun methods (Fig. 3), as has been widely reported for many different transgenes (33, 35–37, 55–57). Transient transfection and in turn GLUT4-GFP overexpression are achieved in only a small percentage (Fig. 3) of muscle fibers (depending on the method), which persists for only a few weeks solely in the muscle subjected to transfection (33). However, the relatively low level and distribution of the transgene expression are advantageous, since disturbances to whole body glucose homeostasis are minimized (33, 37). Conversely, a strong promoter (such as the CMV promoter) is commonly used to achieve adequate transfection levels for experimental measures, which introduces the risk of excessive GLUT4-GFP expression within individual fibers that are transfected, potentially leading to the formation of inclusion bodies or other changes that may alter GLUT4 regulation. The gene gun method primarily transfects muscle fibers on the surface of a given muscle and has a low transfection efficiency (10–200 fibers), but the location of the transfected fibers is ideal for intravitral imaging by confocal microscopy (33). Another advantage of techniques that achieve only a low transfected efficiency is that single GLUT4-GFP-expressing fibers can be visualized by high magnification imaging, which are flanked by “dark” nontransfected fibers. Therefore, this allows for better definition of the sarcolemma of the muscle fiber and reduces interference from fluorescence that may originate in neighboring GLUT4-GFP-expressing fibers as in GLUT4-GFP transgenic animals, where all fibers express the transgene.

Since any animal can be transfected with GLUT4-GFP or other GFP-tagged proteins, this technique can easily be combined with existing transgenic animals. Thereby, changes in GLUT4-GFP traffic could be assessed in the absence (or overexpression) of specific kinases or other metabolic proteins. Alternatively, transfecting muscle cells (cardiomyocytes or muscle fibers) after isolation (i.e., ex vivo) could be achieved; however, this approach may be problematic, since transgene expression requires several days for production of optimal (or possibly measurable) levels of the protein that the transgene encodes, which introduces several problems including survival, viability, denervation, and dedifferentiation of the muscle preparation.

In Situ Detection of Tagged GLUT4

GLUT4-GFP translocation dynamics have been measured in situ in living animals (Fig. 3) (33, 35–37); however, this methodology is complicated by a number of factors. Movements of the anesthetized animal originating from respiration, blood flow, and potential muscle twitching are factors that can make it very difficult to obtain focused images with identical x,y,z coordinates, which is required to track GLUT4-GFP vesicles with in the size range of 1–2 μm over times that can range from a few minutes to 260 minutes (35, 37). In fact, it is easier to continuously track small structures with frequent...
imaging (images obtained every 0.5–2 seconds) compared with images captured less frequently (every 15–60 seconds) due to the movement complications in vivo affecting the optical position of vesicle structures and in turn making it harder to track them with longer time between captured images. Furthermore, the environment surrounding the imaged muscle fiber in the animal is inferior regarding optical light transmission compared with glass slides that are commonly used for in vitro conditions. Capillaries and muscle fibers in cell layers above can be significant obstacles in the optical path and scatter light, thereby reducing the signal to noise, potentially creating images that are dim with a lower resolution (especially the resolution along the z-axis) (60). Gene gun transfection methods utilize gold particles, and if any gold particles remain on the muscle surface after transfection, the image quality can further deteriorate by the light scattering caused by the remnant metal. Generally, only fibers within 100 μm of the muscle surface can be imaged (using confocal microscopy), but multiphoton technology allows for the imaging of muscle fibers deeper within the muscle if desired. Penetration depths of 700–1,000 μm have been demonstrated using multiphoton microscopy in rat brain tissue in situ, a less light-scattering tissue compared with muscle (60). GLUT4-GFP makes it possible to follow the protein movements of GLUT4 in situ, since no cofactors or antibodies are required to visualize GFP except excitation light. However, since the detection is dependent on light excitation, the consequence is that detection is limited by the resolution of light. This means that GLUT4-GFP actually inserted in the plasma membranes cannot be separated from GLUT4-GFP directly inserted into surface membranes of freshly isolated intact muscle fibers. GLUT4-GFP inserted into membranes in muscle homogenates from freshly isolated muscles.

Despite these obstacles, the noninvasive imaging of GLUT4-GFP in situ in mature muscle fibers provides several advantages. Any potential damage from muscle excision and muscle fiber isolation are completely avoided, since it only requires the skin to be gently opened, exposing the muscle...
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surface (34). The muscle fibers in situ are attached to tendons, to other fibers, and to capillaries, thereby ensuring maintenance of passive stretch to the muscle fibers. Furthermore, fibers within the muscle are exposed to a plethora of hormones, peptides, and proteins that are present in interstitial fluid in addition to the constant neural innervation. It is possible that these factors keep the T-tubule network fully dilated, since diffusion of a fluorescent dye like sulforhodamine B into T-tubules of isolated fibers (+20 min) occurs 10–20 times slower in isolated mouse muscle fibers in vitro (32, 64) compared with in vivo conditions (1–2 min) (37). This potential partial collapse of the T-tubules provides a potential explanation for the fact that glucose transport (and GLUT4 translocation) measurements obtained in muscle in vivo tend to be higher than measurements in vitro (4). The fact that GLUT4 translocation can be measured within the context of intact physiological parameters before, during, and after applying a stimulus (e.g., insulin stimulation) makes it an attractive model to access changes in GLUT4-GFP traffic (Fig. 3). This also extends to intact pathophysiological conditions of the various models of type 2 diabetes and insulin resistance, in which GLUT4-GFP traffic studies may ultimately identify factors or relationships that are missing using in vitro models (36).

Intravital Imaging of GLUT4 Traffic

To date, intravital imaging of GLUT4-GFP translocation has been achieved under basal and insulin-stimulated conditions (33, 37), which has confirmed previous observations (from immunostaining of mature muscle fibers) that GLUT4 resides in larger and smaller vesicular structures and translocates from these vesicular stores to the sarcolemma and T-tubules upon insulin stimulation (51, 61).

In addition, intravital imaging has added a new layer of detail since it has been shown that insulin signaling at the level of PI 3-kinase activation and, in turn, GLUT4 vesicle depletion and GLUT4 translocation is compartmentalized. Initially, as insulin arrives at the sarcolemma, local activation of phosphoinositol triphosphate kinase (PI3K) activation ensues [measured as a local increase in GFP fluorescence via a GFP-linked phosphoinositol triphosphate (PIP3) receptor (a PH domain-containing protein)] followed by a local GLUT4 recruitment and translocation. Within the next minutes, as insulin slowly diffuses into the T-tubules, there was a progressive PI3K activation and in turn GLUT4 translocation to the T-tubules (37) (Fig. 4A). The importance of the T-tubules has been further highlighted by intravital imaging in animal models of insulin resistance. It was demonstrated that insulin signaling at the level of PI3K and GLUT4 translocation were specifically reduced in the T-tubules compared with sarcolemma during insulin resistant conditions such as after 12 weeks of high fat feeding or 10 days after muscle denervation (36) (Fig. 4B). In parallel, indirect techniques have shown impairment of the insulin-stimulated GLUT4 translocation to the transverse tubular network, but not the sarcolemma, as determined by subcellular fractionation in streptozotocin-induced diabetic rats (12). Intravital imaging has also shown that the specific reduction in signaling and translocation in the T-tubules after high-fat feeding was not related to reduced accessibility of interstitial fluid, as dye diffusion into the T-tubules was shown to be normal (36) (Fig. 4B).

In contrast, insulin stimulation of L6 myotubes has shown PI3K activation to be present only at the plasma membrane proper (corresponding to the sarcolemma) (48). However, insulin stimulates reorganization (or ruffling) of the surface membrane of the L6 myotubes associated with actin remodeling and PIP3 production, ultimately leading to an extension of the plasma membrane into the cell (48). It has so far been reported that, in mature skeletal muscle, cortical actin is required for insulin-stimulated GLUT4 translocation and glucose uptake (6); however, the actin dependence of insulin-induced GLUT4 translocation (and glucose uptake) has not yet been linked to dynamic actin remodeling in mature skeletal muscle fibers. Therefore, understanding and measuring potential actin remodeling and surface membrane reorganization induced by insulin (or other stimuli) in skeletal muscle is important to investigate using the imaging techniques described in this review.

Intravital imaging has provided new information about GLUT4 traffic during basal vesicular recycling and during insulin-stimulated translocation and reinternalization (35, 37). By use of intravital imaging it was found that the majority of GLUT4-GFP vesicles (that were >1 μm in diameter) did not undergo significant movement in the absence of insulin (35). Use of intravital photobleaching recovery of GLUT4-GFP fluorescence revealed that, during basal and insulin-stimulated translocation and steady-state recycling, only GLUT4-GFP vesicles located near the sarcolemma underwent significant movement along the surface from the perinuclear GLUT4-GFP depots (35). In contrast, the majority of GLUT4-GFP vesicles near the T-tubules were stationary and gradually depleted through microvesicles (10 μm) in their local position during insulin stimulation (35). This is in line with previous studies demonstrating that, when microtubules were depolymerized in mature muscle in vitro, no effect was observed on insulin- or contraction-mediated glucose transport (1). Highlighting the difference between mature muscle and in vitro cultures are findings in models such as 3T3-L1 adipocytes, where a significant degree of GLUT4-GFP movements was associated with microtubulin, and when these microtubulin structures were depolymerized the GLUT4-GFP translocation and glucose transport were reduced by 40% (17).

Thus, intravital imaging studies have shown a compartmentalization between the sarcolemma and the T-tubules that differ in the kinetics at the level of PI3K activation and GLUT4 traffic. It is hard to compare directly these results with results obtained in vitro due to the difference in maturity compared with fully differentiated muscle.

In regard to the other major stimuli of GLUT4 translocation, no studies have so far investigated muscle contraction-induced changes in GLUT4 traffic kinetics in mature muscle with high spatial-temporal resolution.

Transgenic Expression of Tagged GLUT4

In the first transgenic approach using a tagged GLUT4 chimera in mature fibers, muscle-specific GLUT4-GFP overexpression was achieved in mice (28). However, those authors performed analysis of GLUT4-GFP only on frozen cryosections from the basal state, after 30 min of insulin stimulation, or after 60 min of treadmill exercise; thus, there was no improvement in time resolution over traditional immunocyto-
chemical techniques (28). That study demonstrated that GLUT4-GFP localized to the same compartments as endogenous GLUT4 and translocated to the sarcolemma and T-tubules after stimulation with insulin or muscle contraction (i.e., exercise) (28). Furthermore, that study demonstrated that overexpression of GLUT4-GFP in all muscles increased the glucose tolerance of the mice.

Muscle-specific transgenic overexpression of GLUT4-GFP with an HA tag in the exofacial membrane loop has been achieved recently, although potential effects on whole body glucose homeostasis were not reported (15). Also, muscle-specific expression of GLUT4-myc in transgenic mice resulted in a threefold increase in GLUT4 levels in muscle and was in turn associated with increased glucose tolerance (54). These findings reveal that both myc- and GFP-tagged GLUT4 can act as a functional glucose transporter within skeletal muscle, and overexpression has a physiological consequence regarding the maintenance of circulating blood glucose levels.

Upon generation of a transgenic animal, there is often no subsequent need for introducing a transgene into muscle, which may limit disturbances to cell homeostasis or induction of immune responses. In addition, given the genetic stability of the transgenic mouse model, a consistent level of overexpression is expected within a muscle (depending on the promoter used) or muscle group, and differences between different cohorts of transgenic animals should be minimized. These factors could reduce variability in measurements and avoid problems associated with measurements made on cells with various magnitudes of transgene expression.

In Vitro Studies on Freshly Isolated Muscle Cells Expressing Tagged GLUT4

In order to bypass the complex imaging problems of performing GLUT4 translocation analysis in situ in living (anesthetized) animals, another approach used by several groups involves the isolation of muscle fibers or cells from transgenic mice expressing a tagged version of GLUT4 (Fig. 3). To date, this has been achieved in mechanically isolated mouse EDL muscle fibers expressing GLUT4-myc (54) and in collagenase-isolated cardiomyocytes expressing HA-tagged GLUT4-GFP (15).
By imaging the GLUT4 translocation in freshly isolated muscle fibers, the problems associated with sample movement and resolution constraints in the animal are avoided. Furthermore, isolated muscle fibers can be imaged in near optimal optical conditions (i.e., better Z resolution), since the muscle fibers are lying as a “single layer” on a glass coverslip, not embedded beneath capillaries, fascia, or other layers of muscle fibers as in situ. Similar to other in vitro experiments, rapid changes to extracellular environment can be achieved. However, avoiding hypoxic conditions and damage during isolation procedures are important considerations. In order to isolate single muscle fibers, collagenase treatment or mechanical dissection has been used (15, 54). However, collagenase treatment can potentially damage muscle fibers, and this treatment has been shown to result in significant disturbances in ion influx and Ca\(^{2+}\) channel activity up to four days after collagenase isolation (9). Therefore, experiments preformed after enzymatic liberation of muscle cells may not fully recapitulate signaling or vesicular traffic events in vivo. Furthermore, the single fiber isolation is very labor intensive, requires a significant amount of time following muscle excision (that can exceed 30–60 min depending on the competence of the technician), and involves detachment from tendons, loss of neural innervation, and loss of circulating factors (blood supply/interstitial fluid). These factors during (and after) isolation could cause several changes important in the muscle fiber metabolism and in turn in the regulation of GLUT4 traffic, including the partial collapse of T-tubules, limiting accessibility of stimuli or antibodies.

Currently, one of the major advantages of isolated muscle cell experiments using GLUT4 constructs with an exofacial tag is the ability to discern between intracellular and membrane-bound/inserted GLUT4 that is directly participating in glucose transport.

Cardiomyocytes expressing HA-GLUT4-GFP have successfully been used to monitor GLUT4 traffic and identified translocation from internal stores to the cell surface in response to insulin, contraction, and hypoxia. Importantly, a distinction between surface levels of exofacial HA-tagged GLUT4-GFP could be made in cardiomyocytes. This revealed that the gradual GLUT4-GFP staining of either sarcolemma or T-tubules was directly correlated with HA antibody staining for HA-GLUT4-GFP inserted into the membranes (15). Furthermore, this study found stimuli-dependent differences in the level of HA-GLUT4-GFP translocation to sarcolemma and T-tubules (15). These kinetic differences in GLUT4 translocation between surfaces is in line with previous intravital GLUT4-GFP studies under normal physiological conditions (35, 37) as well as during insulin resistant states (36).

A recent study parallels the cardiomyocyte work in small bundles of isolated skeletal muscle fibers from a different line of transgenic mice that overexpress myc-tagged GLUT4 (54). It was demonstrated that the exofacial myc epitope within GLUT4 could be detected in nonpermeabilized EDL muscle fiber segments mechanically isolated from these mice. Immunofluorescence-based detection of surface GLUT4-myc in these fibers appeared as small punctae, which was visually different from the characteristic banding pattern (i.e., cross-striations juxtaposed with T-tubules) observed following detection of GLUT4-myc in permeabilized fibers (Fig. 2). The punctate immunofluorescence staining of surface GLUT4-myc in EDL fibers from these transgenic mice was also visually different from the typical cross-striations observed in cardiomyocytes isolated from HA-GLUT4-GFP transgenic mice, where detection was achieved using a smaller Fab anti-HA antibody or with GFP detection. Unfortunately, Fab anti-myc antibodies did not provide useful insight into EDL fibers from mice that overexpress GLUT4-myc. Given these results, it can be concluded that the detection of surface GLUT4-myc in EDL muscles fibers in this study was restricted to the sarcolemma, and the T-tubules remained inaccessible to staining. One possibility is that the T-tubules are more sensitive to collapse during or after mechanical isolation of skeletal muscle fibers compared with cultured cardiomyocytes. Other possibilities for this difference may include the quality of myc vs. HA antibodies and differences in cardiac vs. skeletal muscle architecture or that T-tubules from skeletal muscle are less accessible than T-tubules from cardiomyocytes and in turn that limits antibody penetration. Nevertheless, the characteristic twofold increase in cell surface (likely sarcolemmal) GLUT4-myc induced by insulin was demonstrated in isolated skeletal muscle fibers from transgenic GLUT4-myc mice (54).

By studying GLUT4 traffic in models that can discriminate between GLUT4 inserted into membranes and intracellular GLUT4, additional experiments could be performed to answer questions regarding how insulin resistance affects the docking and fusion of GLUT4 vesicles in various membranes. By using these models, it would also be possible to pinpoint whether the reduced GLUT4 translocation to T-tubules during insulin resistance is a product of changes in either the exocytic or the endocytic arms of GLUT4 traffic. Future experiments may reveal the possibility of achieving exofacial labeling of GLUT4 inserted into surface membranes of skeletal muscle fibers in vivo or in situ using a hindlimb perfusion model.

**Affinity Photolabeling Techniques**

In contrast to antibody-mediated detection of GLUT4 (or tagged GLUT4) and GLUT4 chimeras that display florescence (GLUT4-GFP), a series of bis-mannose (or glucose) photoaffinity probes has been developed to monitor GLUT4 traffic in various systems and tissues. A detailed description of the development and characteristics of such probes is beyond the scope of this review and can be found elsewhere (21). A major advantage of affinity photolabeling techniques using these probes is the determination of cell surface levels of glucose transporters that are poised to transport glucose in intact cells and tissues. By infusion of a photolabel followed by isolation of the target tissue (43) or, alternatively, exposure of isolated cells/tissues to a photolabel in vitro (53), the muscle tissue is exposed to the tags (Fig. 3). These probes are cell impermeable and do not have access to intracellular glucose transporters, thus giving an accurate estimation of GLUT4 recruitment from intracellular stores. Therefore, following various stimuli, high-energy (UV) cross-linking of the probe to glucose transporters labels only those that are inserted into cell surface membranes, exposed to the probe, and available to transport glucose. Following biochemical techniques to isolate and detect specific glucose transporter isoforms that are labeled with the probes, cell surface GLUT4 can eventually be determined (Fig. 3). A disadvantage of this labeling technique is that the accurate estimation of GLUT4 by imaging techniques is not possible.
due to the photolabel binding to both GLUT4 and GLUT1 isoforms (62). Furthermore, the ultrastructure of muscle (including clear distinction between sarcolemma and T-tubules) is lost during tissue homogenization, and contamination between membrane pools can arise during the subsequent subcellular fractionation steps, which is complicated by the lack of a proper GLUT4 standard (39, 53).

Affinity photolabeling has been successfully used to determine GLUT4 translocation in many systems including skeletal muscle and the heart (43, 62). Unfortunately, visualization of the affinity photolabels bound to glucose transporters in skeletal muscle is limited. These probes appear to penetrate into T-tubules and successfully label both the sarcolemma and T-tubular GLUT4 pools in rodent muscle (13). Similar to other models and assessments of glucose transport, GLUT4 translocation in skeletal muscle induced by insulin was also shown to be additive with muscle contraction when determined by affinity photolabeling techniques (41). Importantly, affinity photolabeling has been used in skeletal muscle to determine parameters of GLUT4 traffic. Affinity photolabeling was successful in identifying divergence in the regulation of exocytosis and endocytosis in adult rat cardiomyocytes induced by insulin vs. energy stress (63). Furthermore, these techniques were applied to rat and human skeletal muscle, where it was demonstrated that a sixfold increase in the exocytosis of GLUT4 was sufficient to explain insulin- (but not metabolic stress-) induced increased levels of surface GLUT4 (26). The insulin-stimulated findings are in parallel to those of previous reports from cultured L6 myotubes (2). In addition, it was found that selective activation of AMPK, a kinase proposed to play a key role in contraction-mediated signaling and consequent increases in cell surface GLUT4 and glucose uptake, did not alter GLUT4 exocytosis in contrast to insulin. AMPK-mediated changes in GLUT4 endocytosis remain controversial on the basis of those measurements. The insulin-stimulated findings are in parallel to previous reports from cultured L6 myotubes (2). Indeed, affinity photolabeling is a powerful technique that will continue to provide insight into the regulation of GLUT4 traffic, and the application to human skeletal muscle is particularly attractive, a feature that has not been achieved by other measurement techniques. In fact, this method has been used to demonstrate that skeletal muscle biopsies from type 2 diabetic patients have defective insulin-stimulated GLUT4 translocation, an effect that could be overcome by AMPK activation (29, 53). The perceived limitations of these methods may reside in the numerous biochemical steps used postlabeling in order to determine the labeled glucose transport isoform (i.e., GLUT4), which may introduce cross-contamination problems and limit temporal resolution and quantitative recovery.

Future Directions

Undoubtedly, the use intravitral measurement of GLUT4-GFP chimeras, antibody-based determinations, exofacial tagged GLUT4, and affinity photolabeling techniques will all be used to assess the regulation of GLUT4 traffic, depending on the limitation of each method and the specific hypotheses tested and experimental goals. Several refinements to these approaches appear warranted. The use of hindlimb perfusion techniques combined with intravitral measurements may allow for nearly immediate changes in the stimuli or inhibitors exposed to muscle fibers and reduce the problem of availability and nonspecific clearance by other organs in the whole body of an anesthetized animal. Also, the use of inducible (and tissue-specific) genetic manipulation in mice is applicable to all of these techniques. If significant barriers to antibody access can be overcome, hindlimb perfusion may also be advantageous for in situ labeling of exofacial tagged GLUT4 in vivo. It is of great interest to develop a method that allows for intravital imaging that can discriminate membrane-inserted (exofacially exposed) GLUT4 that is poised to transport glucose from internal stores, which may reside very close to the membrane in skeletal muscle fibers. Alternatively, improvements in light resolution together with advancements in microscopy, e.g., by implementing new nanoscope technology, could potentially bypass the 200-nm limit of light resolution for intravital imaging and thereby separate structures in the electron microscope range (∼5–20 nm) in vivo (12). Techniques such as improved FRET (fluorescence resonance energy transfer) analysis may also yield methods that allow for the discrimination of GLUT4 near vs. inserted into surface membranes during intravitral imaging of GLUT4-GFP chimeras. Alternatively, the development of probes that allow for the measurement of changes in fluorescence (of adequate magnitudes) upon GLUT4 insertion into muscle fiber membranes would be advantageous. Taken together, it is clear that monitoring GLUT4 (or other proteins) traffic in mature muscle is just beginning.

GRANTS

This work was supported by the Weimann Foundation; the Faculty of Health Sciences, University of Copenhagen; the Beckett Foundation; and the Danish National Research Foundation. J. D. Schertzer is supported by the Canadian Institutes of Health Research and the Canadian Diabetes Association.

DISCLOSURES

No conflicts of interest are reported by the authors.

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