GLUT-3 expression in human skeletal muscle

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Stuart, Charles A., Gary Wen, Bi-Hung Peng, Vsevolod L. Popov, S. David Hudnall, and Gerald A. Campbell. GLUT-3 expression in human skeletal muscle. Am J Physiol Endocrinol Metab 279: E855–E861, 2000.—Muscle biopsy homogenates contain GLUT-3 mRNA and protein. Before these studies, it was unclear where GLUT-3 was located in muscle tissue. In situ hybridization using a mid-molecule probe demonstrated GLUT-3 within all muscle fibers. Fluorescent-tagged antibody reacting with affinity-purified antibody directed at the carboxy-terminus demonstrated GLUT-3 protein in all fibers. Slow-twitch muscle fibers, identified by NADH-tetrazolium reductase staining, possessed more GLUT-3 protein than fast-twitch fibers. Electron microscopy using affinity-purified primary antibody and gold particle-tagged secondary antibody showed that the majority of GLUT-3 was in association with triads and transverse tubules inside the fiber. Strong GLUT-3 signals were seen in association with the few nerves that traversed muscle sections. Electron microscopic evaluation of human peripheral nerve demonstrated GLUT-3 within the axon, with many of the particles related to mitochondria. GLUT-3 protein was found in myelin but not in Schwann cells. GLUT-1 protein was not present in nerve cells, axons, myelin, or Schwann cells but was seen at the surface of the peripheral nerve in the perineurium. These studies demonstrated that GLUT-3 mRNA and protein are expressed throughout normal human skeletal muscle, but the protein is predominantly found in the triads of slow-twitch muscle fibers.

GLUT-3; human muscle; immunofluorescence; in situ hybridization

OF THE FOUR ISOFORMS of facilitative glucose transporters that have been cloned and characterized, three are found in skeletal muscle (1, 26, 27). These are called GLUT-1, GLUT-3, and GLUT-4, based on the order in which they were cloned. GLUT-1 is also called the erythrocyte glucose transporter and was originally called the HepG2 transporter. GLUT-3 is called the brain glucose transporter because of its high level of expression in the central nervous system. GLUT-3 was also known as the human fetal muscle glucose transporter after the tissue from which it was first cloned. GLUT-4 is the insulin-responsive glucose transporter and is also known as the muscle-fat glucose transporter since it is almost exclusively expressed in these two tissues. GLUT-2 has its highest expression in the hepatocyte and is known as the liver glucose transporter. GLUT-2 is also found in the β-cell of the pancreas and is involved in sensing ambient glucose concentrations (10). Although GLUT-5 has ~40% amino acid identity and 50–60% similarity to the other four isoforms (2), it only transports fructose (1). GLUT-5 is predominantly expressed in the epithelial cells of the small intestine but is also expressed in skeletal muscle plasma membrane (6).

Both GLUT-1 and GLUT-3 mRNA have been detected in most tissues (2, 14), but the presence of significant amounts of GLUT-3 in human muscle cells has been questioned (14, 26, 29). It has been suggested that the GLUT-3 present in muscle might be from contaminating neural or vascular elements (14, 26, 29). In the current studies, we sought to determine the cellular and subcellular localization of GLUT-3 in human muscle samples.

MATERIALS AND METHODS

Materials. Antisera against human GLUT-1 and GLUT-3 were purchased from Alpha Diagnostic International (San Antonio, TX). These affinity-purified antibodies were raised in rabbits against the 13 and 12 carboxy-terminal amino acids deduced from the human GLUT-1 cDNA and human GLUT-3 cDNA, respectively. Rabbit IgG was purchased from Sigma (St. Louis, MO).

Muscle specimens were obtained from normal volunteers. Percutaneous needle biopsies were obtained from vastus lateralis muscle as previously described (5). Each biopsy was performed after an overnight fast and 2 h of quiet recency.

Two additional specimens were obtained at autopsy. One autopsy specimen that was used extensively as a reference specimen was from gastrocnemius muscle of a young adult trauma victim. A second autopsy provided a sample from a spinal nerve for use in electron microscopic localization of GLUT-3 and GLUT-1.

In situ hybridization of GLUT-3 glucose transporter riboprobes in muscle tissue. Tissue specimens were immediately snap-frozen in liquid nitrogen and later were cut in 10-μm-thick frozen sections using RNase-free knives and other materials. The probe for GLUT-3 was digoxigenin labeled using a kit from Boehringer Mannheim and a pBSM13 plasmid containing a 256-bp Sac I/EcoR I GLUT-3 cDNA fragment from pBS-MGT3 as previously described for 32P labeling (31).

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The orientation of the cDNA was such that, after appropriate linearization, T3 RNA polymerase generated the antisense riboprobe and T7 produced the sense riboprobe that was used for nonspecific labeling of the sections. Prehybridization and hybridization were performed according to the techniques described with the digoxigenin-labeling kit and published by Dijkman and co-workers (7). After 16 h incubation at 37°C and subsequent washing, the sections were counterstained with 1% methylene green.

**Immunohistochemistry and immunofluorescence of glucose transporters in human skeletal muscle.** Fresh, unfixed muscle biopsy specimens were frozen for 30 s in isopentane cooled to −150°C with liquid nitrogen, embedded in optimum-cutting temperature compound on cryostat chucks, and later sectioned at 12 μm thickness on a cryostat at −17°C. Cryosections were mounted on slides coated with poly-L-lysine and fixed for 5 min in 4% paraformaldehyde in PBS at room temperature. Sections to be reacted using the alkaline phosphatase system were preincubated in 1% acid alcohol (HCl in ethanol) to block endogenous alkaline phosphatase. The entire immunostaining procedure was carried out at room temperature using the following reaction sequence: primary antibody [rabbit polyclonal anti-human (h) GLUT-3] in dilution buffer (Biomeda) for 45 min; PBS wash three times; biotinylated anti-rabbit IgG (Sigma) diluted 1:80 for 15 min; PBS wash three times; avidin-conjugated alkaline phosphatase (Sigma) 1:80 for 15 min; PBS wash three times; substrate for alkaline phosphatase (fast red tetrazolium reductase/naphthol in Tris buffer; Sigma); hematoxylin (Richard-Allan Scientific, Kalamazoo, MI) counterstain for 15 s; water wash three times followed by placement of a cover slip with aqueous mounting medium (Biomed, Foster City, CA).

For immunofluorescence, sections were incubated with primary antibody (affinity-purified rabbit polyclonal anti-hGLUT-3) in dilution buffer (Biomed) for 45 min at room temperature and then were incubated with FITC-conjugated goat anti-rabbit IgG antibody (Sigma) for 15 min at room temperature. Slides were viewed using a fluorescence microscope (Nikon Optiphot) with a blue (B-2A) filter cube, and photographs were taken using the attached Nikon microscope camera.

Nonspecific staining was determined by incubating control sections with rabbit IgG at the same concentration as the affinity-purified anti-hGLUT-3 antibody.

A mitochondria-specific stain was used to determine the pattern of fiber types in the normal muscle sections. NADH-tetrazolium reductase was identified in sections using the method of van Wijhe et al. (33), according to the details described by Dubowitz (9). Fibers strongly positive for NADH-tetrazolium reductase were designated type 1 oxidative (slow twitch; see Ref. 9).

Image analysis was performed using ImageQuant software from Molecular Dynamics (Sunnyvale, CA). Photomicrographs were digitized using a ScanJet 4c flatbed scanner from Hewlett Packard (Boise, ID).

**Immunogold labeling of glucose transporters in tissue sections.** For postembedding immunoelectron microscopy, freshly obtained muscle biopsy material was immediately placed in a mixture of 2.5% formaldehyde, 0.5% glutaraldehyde, and 0.03% trinitrophenol in 0.5 M cacodylate buffer, pH 7.3; cut into smaller pieces (~1 mm³); and fixed for 2 h. Autopsy-obtained tissues were either placed directly in fixative or were frozen in liquid nitrogen and later were placed in fixative. After being washed in the buffer, the tissue was stained en bloc with 1% uranyl acetate in 0.1 M maleate buffer, pH 5.2, dehydrated in 50 and 75% ethanol, and embedded in medium-grade LR White resin (Structure Probe, West Chester, PA). Ultrathin sections were cut with a Sorvall MT-6000 ultramicrotome and were placed on nickel Formvar- and carbon-coated grids. They were incubated first in the blocking buffer containing 0.1% BSA and 0.01 M glycine in 0.1 M Tris-buffered saline (TBS), then for 1 h at room temperature, and then overnight at 4°C with primary antibody (affinity-purified rabbit polyclonal anti-hGLUT-3 antibody). The grids were washed in blocking buffer and reacted with secondary antibody, conjugated to 15 nm gold particles (goat anti-rabbit IgG [H+L], AuroProbeEM GAR G15; Amersham Life Science, Arlington Heights, IL) diluted 1:20 in 1% BSA in 0.1 M TBS for 1 h at room temperature. After being stained with uranyl acetate and lead citrate, the grids were examined in a Philips 201 electron microscope at 60 kV with instrumental magnifications from 3,000 to 50,000. To determine nonspecific labeling, GLUT-3 immunizing peptide was added to the affinity-purified antibody (0.1 μg/ml) at a final concentration of 1 μg/ml.

**RESULTS**

**Distribution of GLUT-3 mRNA in normal human skeletal muscle as reflected by in situ hybridization.** Digoxigenin-labeled probe was prepared using a kit from Boehringer Mannheim and a pBSM13 plasmid that contained a 256-bp fragment of GLUT-3 cDNA as described in MATERIALS AND METHODS. The micrographs shown in Fig. 1 are high-power views of sections of a muscle specimen. The blue dots identify the location of hybridized probe, indicating widespread expression throughout the section (A). There was no blue staining in control sections incubated with digoxigenin-labeled sense strand (B). All fibers were positive for GLUT-3 mRNA by this technique, but some of the fibers showed a lower density of blue dots. This difference in staining may be analogous to that seen with the immunofluorescent studies described below that demonstrated more GLUT-3 protein in slow-twitch fibers.

**Immunofluorescence and immunohistochemical localization of GLUT-3 in human skeletal muscle.** The affinity-purified, polyclonal rabbit anti-human GLUT-3 antibody was used as primary antibody in studies of the normal muscle sections by techniques described above. Secondary antibody in the study shown in Fig. 2 was labeled with FITC, and the resulting fluorescence was photographed using a Nikon Optiphot scope. The points of bright fluorescence in the control section (B) may be from lysosomes or lipofuscin autofluorescence (25). Figure 2A shows that all muscle fibers are positive for GLUT-3 protein, but a substantial number have a much lower signal. Image analysis revealed a ratio of intensity between the two fiber types to be 2.9 in these sections. Alternate sections stained for NADH-tetrazolium reductase (9) revealed staining patterns similar to that shown in Fig. 2, indicating type 1 oxidative fibers contained more GLUT-3 protein. Figure 3 shows two consecutive sections of normal muscle, with Fig. 3A displaying GLUT-3 immunofluorescence and Fig. 3B showing the same pattern of fibers staining positive for the NADH-tetrazolium reductase. The type 2 fibers exhibit lower mitochondrial marker enzyme and lower GLUT-3 signal.
Ultrastructural localization of GLUT-3 protein within muscle cells from normal subjects. The light microscopic studies using immunofluorescence and immunohistochemistry demonstrate that GLUT-3 protein is found within muscle fibers and that its expression is higher in type 1 oxidative fibers. Because GLUT-4 translocation in muscle is predominantly to transverse tubules (8, 23, 28), we considered that the GLUT-3 protein association with plasma membrane markers in muscle (32) might also be related to transverse tubules. Thus we determined the subcellular distribution of GLUT-3 protein within muscle cells using electron microscopy and antibodies labeled with gold particles. Figure 4 displays an electron micrograph of normal human muscle probed with affinity-purified rabbit polyclonal anti-hGLUT-3 antiserum. The gold particles indicated by the arrows represent specific labeling of transverse tubule structures. These studies found almost all GLUT-3 was associated with triads and transverse tubules. Only a few grains were associated with sarcolemma. Similar results were seen in a second control specimen and in one autopsy specimen. Adding 150-fold molar excess immunizing peptide to the affinity-purified anti-hGLUT-3 antibody completely eliminated gold particles (not shown).

GLUT-3 associated with nerves within muscle biopsies. The GLUT-3 protein signal is clearly present within the myocyte but appears much stronger in the nerves that are seen within the muscle sections. In the central nervous system, GLUT-3 is found only within the nerve cells (22). GLUT-3 is associated with nerve cell bodies but is expressed at higher levels in axons (22). To determine if similar associations are also characteristic of the peripheral nerves found in muscle, we examined muscle sections both with light microscopy and electron microscopy. For the light microscopy studies shown here, conditions were adjusted to optimize nerve staining that decreased muscle cell staining. Figure 5 shows examples of the immunohistochemical studies. Figure 5, A and B, shows a nerve cut transversely, and Fig. 5, C and D, shows longitudinal sections through nerves. Figure 5, A and C, shows red color for GLUT-3 protein recognized by the affinity-purified antibody. Figure 5, B and D, contains control

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**Fig. 1.** GLUT-3 mRNA localization in human skeletal muscle using in situ hybridization of a digoxigenin-labeled probe. A: high-power view of a muscle section incubated with an anti-sense probe. B: similar section from the same muscle that was incubated with a sense probe to show nonspecific staining. Background stain was nuclear fast red. The magnification is the same for both A and B. Bar indicates 50 μm.

**Fig. 2.** Normal human muscle GLUT-3 protein distribution as determined by immunofluorescence. Fresh normal human muscle was processed as described in MATERIALS AND METHODS. All muscle fibers in A show specific labeling with the fluorescent probe. B: section that was incubated with buffer only [no affinity-purified anti-human (h) GLUT-3 polyclonal antibody]. The nonspecific points of fluorescence may represent lipofuscin or lysosome autofluorescence. Magnification is the same in A and B. Bar represents 100 μm.
sections incubated with nonimmunized rabbit IgG. Figure 5A suggests that the axons are positive for GLUT-3, and Fig. 5C suggests that some of the supporting cells may also be positive for GLUT-3.

To clarify what specific cells of peripheral nerves contain GLUT-3, we obtained a spinal nerve at autopsy and evaluated the ultrastructural localization of GLUT-1 and GLUT-3 proteins using immunogold particles. Figure 6 displays electron micrographs with specific labeling of GLUT-3 protein (A) and of GLUT-1 (B). GLUT-1-associated gold particles were found in close proximity to the perineurium but not in the nerve axon or Schwann cells. No capillaries were identified in these sections. GLUT-3 label was seen within the nerve axons and in association with myelin. The presence of myelin-associated gold particles suggests that Schwann cells may possess GLUT-3, although no particles were seen directly overlying Schwann cell bodies.

DISCUSSION

Human skeletal muscle contains mRNA for GLUT-1, GLUT-3, and GLUT-4. Whether the amounts of GLUT-1 or GLUT-3 present were physiologically important in humans has been questioned (1). GLUT-1 is present at the cell surface in rat muscle and was considered to mediate the basal glucose uptake (2). GLUT-3 is expressed most abundantly in brain and neural tissue (1, 2, 14, 26, 27). Its presence in muscle has been attributed to the contamination of muscle specimens with the nerves that innervate the fascicles (14, 26, 29).

Nerves contained in muscle biopsies display strong signals for GLUT-3 mRNA and GLUT-3 protein by in situ hybridization and immunohistochrometry, but nearly 90% of the signals are found in muscle cells. A similar pattern of GLUT-1 distribution as determined by immunofluorescence was shown by Handberg and co-workers (17) in rat muscle. These investigators found a strong GLUT-1 signal in perineural cells but none in vascular endothelium (17).

Red muscle fibers have higher glucose uptake than white fibers in the basal state and in response to
insulin (3, 18). Three previous reports agree that GLUT-4 is expressed at higher levels in red (type I oxidative) fibers (13, 21, 24), but one of these reports differs on whether or not GLUT-1 is increased in red fibers (21, 24). Kern and co-workers (21) evaluated mRNA and protein abundance in red and white muscle from male Sprague-Dawley rats (21). In homogenates from these muscles, GLUT-4 protein was fivefold

![Fig. 5. Muscle sections showing GLUT-3 protein associated with peripheral nerve. A-D are of similar magnification and staining except that A and C were incubated with affinity-purified antibody against the carboxy-terminal 12 amino acids from human GLUT-3 and B and D were incubated with nonimmune rabbit IgG as first antibody. A and B show transverse cuts of a peripheral nerve axon, and C and D show nerves cut tangentially. Specific staining associated with the anti-hGLUT-3 antibody is indicated by the red color. Arrows in A and C point to individual cells among those positive for GLUT-3 protein.](image)

![Fig. 6. Electron micrograph of human spinal nerve from an autopsy. A: myelinated nerve axon incubated with affinity-purified anti-hGLUT-3 antibody and subsequently incubated with a gold particle-labeled goat anti-rabbit second antibody. Arrows mark gold particles. Ax, axon; M, marks surrounding myelin. B: section incubated first with affinity-purified anti-hGLUT-1 antibody. Specific labeling of GLUT-1 was only seen at the periphery of the nerve in association with the perineurium. GLUT-3 labeling was present only in the axons or associated with myelin. Arrows point to gold particles. Bars indicate 1 μm.](image)
higher, and GLUT-4 mRNA was twofold higher in red fibers. They did not find a difference in the expression of GLUT-1 mRNA or protein in the red and white fibers. Goodyear et al. (13) compared cytochalasin B binding in male Sprague-Dawley rat red and white gastrocnemius muscle-derived plasma membranes. Cytochalasin B binding was 2.4-fold higher in membranes from red fibers. Exercise and insulin treatment increased cytochalasin B binding in the plasma membranes from both red and white fibers (red 40%, white 100%; see Ref. 13). Immunoblots showed that both GLUT-4 and GLUT-1 protein were higher in red than white muscle (13). Mareette and co-workers (24) evaluated the expression of GLUT-1 and GLUT-4 in rat red and white muscle using cytochalasin B binding, immunoblotting, and immunofluorescence. These investigators found that both GLUT-1 and GLUT-4 proteins were higher in red muscle-derived plasma membranes. Most of this difference was due to increased expression of both at the plasma membrane. Indirect methods for absolute quantification suggested that plasma membranes from both red and white muscles contained fourfold more GLUT-4 than GLUT-1 in the basal state (after an overnight fast; see Ref. 24).

We speculate that previous reports of undetectable GLUT-3 mRNA (11, 20) or protein (16, 29) in human muscle were due to the assay of muscle samples from autopsies containing degraded mRNA and protein. Not all autopsy-derived muscle specimens have low GLUT-3 mRNA and protein. Our recent report (31) and the report of Yano and co-workers (34) show abundant GLUT-3 mRNA and protein signals from some human muscle specimens obtained postmortem. We have recently shown that several mRNA species are rapidly degraded in muscle at room temperature and that GLUT-3 protein disappears at an even faster rate than GLUT-3 mRNA (31). Muscle specimens at autopsy are thus likely to dramatically underestimate the quantity of mRNA or protein that is actually present in normal muscle tissue.

Human cardiac myocytes contain GLUT-3 protein. Grover-McKay et al. (15) have recently reported immunohistochemical demonstration of GLUT-3 protein within myocytes obtained from myocardial biopsies from one heart donor and seven explanted hearts from recipients. They also demonstrated that GLUT-3 protein was present in heart muscle from an adult autopsy and six human fetuses. This group of investigators used techniques similar to ours and the same type of affinity-purified polyclonal antibody to demonstrate strong immunohistochemical signals by light and electron microscopy (15). They did not report any evaluation of skeletal muscle.

Our immunofluorescence studies give the appearance that GLUT-3 protein is distributed throughout the muscle cell rather than exclusively at the cell surface. This appears to be due to GLUT-3 being distributed in the transverse tubules of the myocyte, similar to what has been shown recently for GLUT-4 (12). Several studies now suggest that GLUT-4 translocated to the “cell surface” by insulin (8, 12, 23) or exercise (8, 28) is actually translocated to transverse tubules.

Katz and co-workers (19) developed transgenic mice with the GLUT-4 gene disrupted. These GLUT-4 null mice exhibited several metabolic abnormalities but did not develop diabetes (19, 30). Fasting blood glucose and insulin concentrations were normal. Fed glucose concentrations and glucose concentrations after an oral glucose challenge were also normal, but fed insulin concentrations were at least fivefold higher than those of the controls (19). The mechanism by which oral glucose is cleared from the blood is unclear. The GLUT-3 isoform has not been evaluated in these animals, but, since GLUT-1 protein was not increased in muscle of the GLUT-4 null mice (30), GLUT-3 might be involved in the compensation for the absence of GLUT-4.

Preliminary reports of GLUT-1 and GLUT-3 expression in muscle biopsies from insulin-resistant subjects show that GLUT-3 mRNA is low and GLUT-1 mRNA is increased compared with normal control subjects (32). GLUT-3 protein in muscle homogenate was decreased. Subcellular fractionation of muscle samples showed that ~85% of GLUT-3 protein was in the plasma membrane marker-enriched fractions for both controls and insulin-resistant subjects (32). These data suggest that basal cell surface glucose transporter content (GLUT-1, GLUT-3, and GLUT-4) may be important correlates of insulin responsiveness.

We conclude that GLUT-3 mRNA and protein are present in normal muscle cells. Our observations suggest that, like GLUT-1 and GLUT-4, GLUT-3 is expressed at a higher level in type 1 oxidative (slow-twitch) muscle fibers. The subcellular localization of GLUT-3 within muscle appears to be predominantly in triads and transverse tubules. This expression and localization within skeletal muscle, coupled with GLUT-3’s lower Michaelis constant for D-glucose (4), suggest that, along with GLUT-1 and GLUT-4, GLUT-3 may play a significant role in providing glucose uptake into muscle.

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