Sarcolemmal FAT/CD36 in human skeletal muscle colocalizes with caveolin-3 and is more abundant in type 1 than in type 2 fibers

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Abstract FAT/CD36 is a transmembrane protein that is thought to facilitate cellular long-chain fatty acid uptake. However, surprisingly little is known about the localization of FAT/CD36 in human skeletal muscle. By confocal immunofluorescence microscopy, we demonstrate high FAT/CD36 expression in endothelial cells and weaker but significant FAT/CD36 expression in sarcolemma in human skeletal muscle. No apparent intracellular staining was observed in the muscle cells. There are indications in the literature that caveolae may be involved in the uptake of fatty acids, possibly as regulators of FAT/CD36 or other fatty acid transporters. We show that in sarcolemma, FAT/CD36 colocalizes with the muscle-specific caveolae marker protein caveolin-3, suggesting that caveolae may regulate cellular fatty acid uptake by FAT/CD36. Furthermore, we provide evidence that FAT/CD36 expression is significantly higher in type 1 compared with type 2 fibers, whereas caveolin-3 expression is significantly higher in type 2 fibers than in type 1 fibers.—Vistisen, B., K. Roepstorff, C. Roepstorff, A. Bonen, B. van Deurs, and B. Kiens. **Sarcolemmal FAT/CD36 in human skeletal muscle colocalizes with caveolin-3 and is more abundant in type 1 than in type 2 fibers. *J. Lipid Res.* 2004.** 45: **603–609.**

Supplementary key words GLUT4 • caveolae • immunofluorescence • fatty acid uptake • lipid binding proteins • endothelial cells • fatty acid translocase

Long-chain fatty acids are an important energy source in skeletal muscle. The cellular uptake of long-chain fatty acids in tissues is thought to be mediated by both passive and facilitated diffusion. Several putative fatty acid transporters have been identified, among them the fatty acid translocase (FAT), which is homologous to human glycoprotein IV or CD36 (1). FAT/CD36 has been found in several tissues with a high metabolic capacity for long-chain fatty acids, such as adipose tissue, heart, and skeletal muscle in rat (1–3) and skeletal muscle in human (4, 5), consistent with its role as a fatty acid binding protein and/or transporter. Furthermore, in mice, when FAT/CD36 was either overexpressed or knocked out, FAT/CD36 was shown to play a role in the cellular uptake of fatty acids (6). It has been suggested from fractionation studies that FAT/CD36 in rat skeletal muscle localizes to both an intracellular pool and the sarcolemma and that FAT/CD36 translocates from the intracellular pool to the sarcolemma in response to muscle contraction and insulin stimulation (7, 8). However, no morphological studies have addressed the localization of FAT/CD36 in human skeletal muscle.

The mechanism(s) by which FAT/CD36 facilitates cellular fatty acid uptake is unknown. Recent data on HepG2 cells suggest that caveolae may play a significant role in the uptake and intracellular trafficking of long-chain fatty acids (9). Caveolae are small 50–100 nm invaginations of the plasma membrane and are present in most tissues (10). Caveolin, which is the marker protein of caveolae and a necessary protein for caveolae formation, exists in three isoforms, of which caveolin-1 is expressed in several cell types such as endothelial cells, fibroblasts, and adipocytes, whereas caveolin-3 is muscle cell-specific (10). The use of caveolin-1 knockout mice indicated that caveolin is important for cellular fatty acid uptake (11). One putative mechanism whereby caveolin could be involved in cellular fatty acid uptake is that caveolae may regulate the function of fatty acid transporters such as FAT/CD36.

In the present study, we have investigated, by confocal microscopy, the localization of FAT/CD36 in human skeletal muscle. Our data show that FAT/CD36 is expressed abundantly in endothelial cells and to a lesser degree in

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sarcolemma, with no apparent intracellular staining for FAT/CD36 in muscle cells. The sarcolemmal FAT/CD36 colocalizes with caveolin-3, suggesting that caveolae may regulate cellular fatty acid uptake by FAT/CD36. We also show that sarcolemmal FAT/CD36 expression is higher in type 1 than in type 2 muscle fibers.

METHODS

Subjects

Six healthy, moderately trained men (age, 25 ± 1 years; height, 1.86 ± 0.02 m; body mass, 80.3 ± 2.9 kg; peak oxygen consumption, 4.4 ± 0.1 l/min) participated in the study. All subjects were fully informed of the possible risks associated with participation in the study and gave their written consent to participate. The study was approved by The Copenhagen Ethics Committee and carried out in accordance with the Declaration of Helsinki II.

Muscle samples

In each subject, a biopsy was obtained from the vastus lateralis muscle under local anesthesia. The biopsy was mounted in embedding medium, frozen in precooled isopentane, and stored at −80°C for subsequent cryosectioning.

Antibodies

Rabbit polyclonal anti-caveolin-1 and -3 antibody (diluted 1:1,600) was obtained from Cayman Chemical. Mouse monoclonal anti-caveolin-3 (clone 26) antibody (diluted 1:400) was from Transduction Laboratories (Lexington, KY). Mouse monoclonal anti-MHC-1 (clone A4.840) antibody (diluted 1:100), which recognizes myosin heavy chain type I (type I muscle fibers) (12), was obtained from the Developmental Hybridoma Bank (University of Iowa). The mouse monoclonal anti-FAT/CD36 (clone MO25) antibody (diluted 1:200) was kindly donated by Dr. Narendra Tandon (Otsuka Maryland Research Institute, Rockville, MD). The rabbit polyclonal anti-GLUT4 (P1) antibody (diluted 1:400) was kindly donated by Dr. Thorkil Ploug (University of Copenhagen, Denmark). Western blotting (13) on skeletal muscle with anti-caveolin-3, anti-FAT/CD36, or anti-GLUT4 antibody results in the detection of a single protein band of the expected size (14) (**Fig. 1**). Secondary fluorescent antibodies (diluted 1:400) were Alexa 488 goat anti-mouse IgG, Alexa 568 goat anti-mouse IgG, Alexa 488 goat anti-rabbit IgG, Alexa 568 goat anti-rabbit IgG, and Alexa 568 goat anti-mouse IgM purchased from Molecular Probes (Leiden, The Netherlands).

Immunofluorescence staining

Transverse or longitudinal cryosections (10 μm) from human vastus lateralis muscle were transferred to glass slides, fixed in methanol-acetone (1:1) at −20°C for 5 min, and allowed to dry at room temperature for 10–15 min. The sections were incubated with blocking buffer [5% goat serum (Invitrogen, Taastrup, Denmark) and 0.2% saponin (Sigma Aldrich, Steinheim, Germany) in PBS] for 1 h at room temperature and incubated with primary antibody diluted in blocking buffer for 1 h at room temperature. Sections were rinsed three times with PBS and incubated with secondary antibody diluted in blocking buffer for 1 h at room temperature. Sections were rinsed three times with PBS and mounted with Fluoromount (Southern Biotechnology).

Confocal microscopy

The muscle sections were analyzed by a Zeiss LSM 510 Meta confocal microscope equipped with 16×/numerical aperture (N.A.) 0.5 and 63×/N.A. 1.4 oil-immersion objectives. Laser lines of 488 and 543 nm were used together with appropriate filter settings. In most cases, the range of the detector system was set so that no pixels were saturated. For visualization of sarcolemmal FAT/CD36, the range of the detector system was set so that no saturation occurred in the sarcolemma but the endothelial signal was saturated. For images of double-labeled sections, the multitrack function was used to ensure complete separation of the red and the green channels. Overview images of MHC-1 and FAT/CD36 on transverse sections were collected using the 16× objective. All other images were collected using the 63× objective. For overview images, a fully open pinhole was used to gather as much signal as possible. For high-magnification images, the pinhole was set to a diameter of 1 airy unit to optimize confocality. No fluorescence signal was detected when muscle sections were incubated only with secondary antibody (data not shown). All images shown are representative of all six subjects.

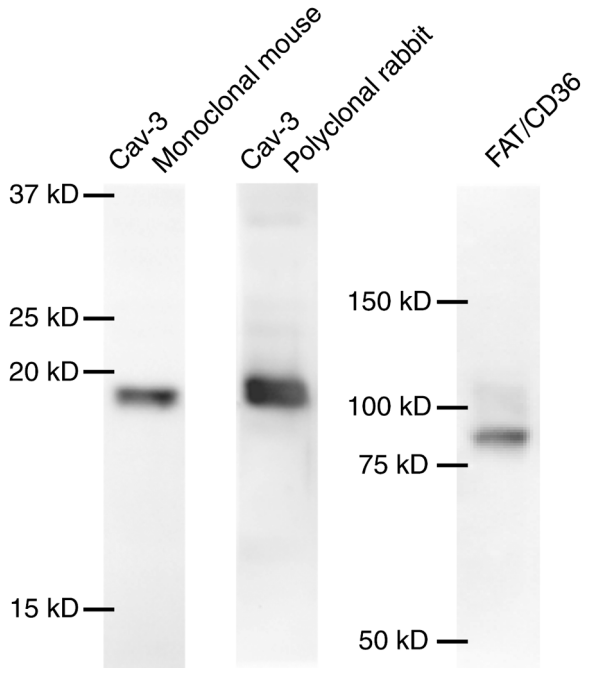

Fig. 1. Representative Western blots of caveolin-3 (Cav-3; mouse monoclonal and rabbit polyclonal) and fatty acid translocase (FAT)/CD36 showing single bands at ~18 and 88 kD, respectively.

Quantification of FAT/CD36 and caveolin-3 expression in sarcolemma

On transverse cryosections from vastus lateralis muscle stained with either anti-FAT/CD36 or anti-caveolin-3, the intensity of the fluorescent signal in sarcolemma was rated (1, 2, or 3) by a person who was blinded regarding the fiber type pattern and origin of the sections. To check interobserver variability, another blinded person also rated the intensity of the sarcolemmal fluorescence signal with similar results. In each subject, ~100 fibers were rated. Fiber type was then determined and the percentage of fibers within each fiber type rated 1, 2, and 3 was calculated.

Statistics

For the quantification of FAT/CD36 and caveolin-3 in sarcolemma, it was determined by paired *t*-test whether there was any difference between type 1 and type 2 fibers in the percentage of fibers given each rating (1, 2, or 3). The level of significance was 0.05.

RESULTS

Distribution of FAT/CD36 in human skeletal muscle

To investigate how FAT/CD36 is distributed in human skeletal muscle, cryosections were immunofluorescently labeled and analyzed by confocal microscopy. As can be seen from **Fig. 2B**, there is a distinct staining of the endothelial cells present between the muscle fibers and a weaker but specific staining of the sarcolemma of the muscle fibers. A similar distribution of FAT/CD36 was seen in cryosections of rat soleus muscle (data not shown). To visualize the sarcolemmal FAT/CD36 more clearly, the detector gain of the microscope was increased to a level at which the endothelial signal was saturated, whereas the sarcolemmal signal was within the linear range of the detector. Fig. 2C shows a close-up of three adjacent muscle fibers using such detector settings. The parallel plasma membranes are clearly stained, showing that the sarcolemma contains FAT/CD36 (Fig. 2C) with very little, if any, intracellular staining. Because of the high detector gain in Fig. 2C, evenly distributed nonspecific staining is apparent. Previous biochemical studies have suggested that a substantial pool of FAT/CD36 is present in an intracellular pool, from which it can translocate to the sarcolemma (7, 8), a mechanism resembling the inducible translocation of GLUT4 from intracellular tubular-vesicular structures to the sarcolemma (15). Therefore, we compared the subcellular distribution of FAT/CD36 with the subcellular distribution of GLUT4. Fig. 2A shows a muscle section stained for GLUT4 using the same detector settings used in Fig. 2B for FAT/CD36. As has been shown by others (16–18), GLUT4 was present both intracellularly and in the sarcolemma. Compared with FAT/CD36, GLUT4 is located to a much higher degree intracellularly (compare Fig. 2B with 2A).

Colocalization of FAT/CD36 with caveolin-3

In longitudinal muscle sections, a few fibers were cut tangentially to the sarcolemma. In such areas, it was possible to obtain images of the surface of the fibers. **Figure 3A–C** show a longitudinal section in which part of a fiber has been cut tangentially, enabling visualization of the sarcolemma. To investigate the localization of FAT/CD36 relative to caveolin-3 in the sarcolemma, such longitudinal sections from human vastus lateralis muscle were double labeled for caveolin-3 and FAT/CD36. As can be seen from Fig. 3D–I, FAT/CD36 (red) and caveolin-3 (green) in the sarcolemma have the same characteristic striated pattern of distribution. It is evident from the merged images (Fig. 3F, I) that there is a high degree of colocalization of caveolin-3 and FAT/CD36 (yellow). When comparing the enlarged images of FAT/CD36 (Fig. 3G) and caveolin-3 (Fig. 3H), it is clear that their distribution is to a very high degree the same (large arrows). GLUT4 has been shown by both confocal microscopy and electron microscopy not to colocalize with caveolin-3 in muscle fibers (16, 17). Therefore, as a control, we double labeled longitudinal sections for caveolin-3 and GLUT4 and examined the distribution in the sarcolemma. As shown by others (16, 17), GLUT4 appeared concentrated in dots at the sarcolemma, most of which were found in caveolin-3-negative areas (Fig. 3J–L, small arrows).

Fiber type-specific sarcolemmal expression of FAT/CD36 and caveolin-3

To investigate whether the expression of FAT/CD36 differs between type 1 and type 2 muscle fibers, we labeled serial transverse cryosections from vastus lateralis muscle from four subjects with either anti-FAT/CD36 or anti-MHC-1 antibody. Subsequently, we evaluated the intensity of the sarcolemmal FAT/CD36 fluorescent signal by assigning each fiber an intensity rating of 1, 2, or 3 in ~100 fibers from each subject. When collecting images from the same subject, the microscope settings were kept constant and the detector gain was set to a level at which the sarcolemmal signal was within the linear range of the detector. From **Fig. 4A, B**, which shows two cryosections in series stained for MHC-1 and FAT/CD36, respectively, it is obvious that sarcolemmal FAT/CD36 expression is higher in type 1 than in type 2 fibers. The variation in sarcolemmal FAT/CD36 expression is even clearer in Fig. 4C, which shows a close-up of Fig. 4B. Figure 4G shows the rel-

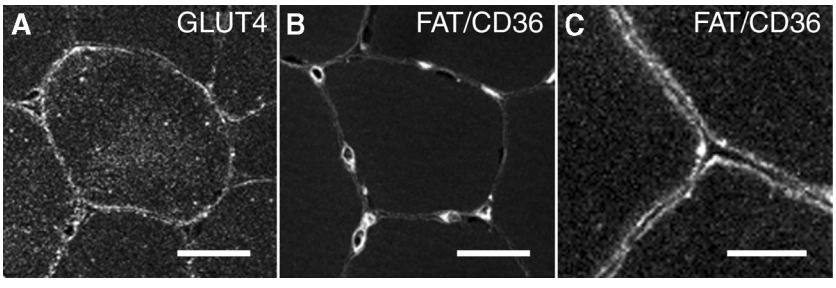

Fig. 2. Distribution of FAT/CD36 and GLUT4 in human skeletal muscle. Transverse cryosections from human vastus lateralis muscle were fixed and labeled with anti-GLUT4 rabbit polyclonal antibody (A) or anti-FAT/CD36 mouse monoclonal antibody (B and C). C shows a close-up of three adjacent muscle fibers. In C compared with A and B, the detector gain of the microscope was increased to a level at which the endothelial signal was saturated, whereas the sarcolemmal signal was within the linear range of the detector. Because of the high detector gain in C, evenly distributed nonspecific staining is apparent. Bars = 30 μm in A and B and 10 μm in C.

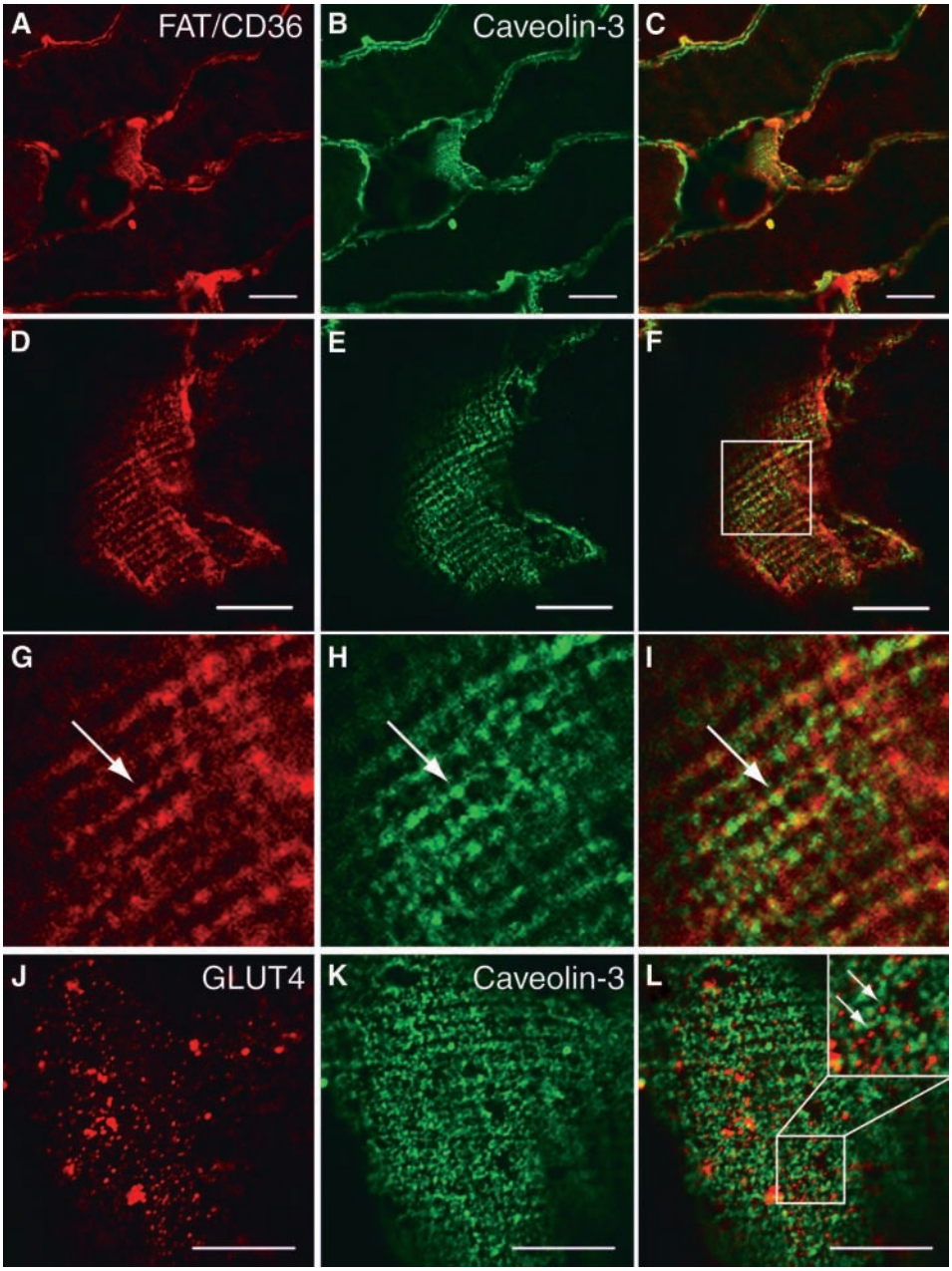

Fig. 3. Colocalization of FAT/CD36 and caveolin-3 in human skeletal muscle. Longitudinal cryosections from human vastus lateralis muscle were fixed and double labeled with either anti-FAT/CD36 mouse monoclonal antibody and anti-caveolin-1 and -3 rabbit polyclonal antibody (A–I) or anti-GLUT4 rabbit polyclonal antibody and anti-caveolin-3 mouse monoclonal antibody (J–L). A–C: Overview of several muscle fibers double labeled for FAT/CD36 (red) and caveolin-3 (green). D–F: Images of the central part of panels A–C showing the surface of a single muscle fiber. G–I: Magnification of the central part of panels D–F. Arrows indicate a spot with a clear colocalization of FAT/CD36 and caveolin-3. J–L: Surface of a muscle fiber double labeled for GLUT4 (red) and caveolin-3 (green). Arrows indicate GLUT4-positive spots in caveolin-3-negative regions. Merged images are shown in C, F, I, and L. Bars = 30 μm in A–C and 10 μm in D–F and J–L.

ative number of fibers rated 1, 2, or 3 for either type 1 or type 2 fibers. As can be seen, there is a marked and significant difference between type 1 and type 2 fibers in the percentage of fibers expressing sarcolemmal FAT/CD36 weakly (rating 1) or abundantly (rating 3).

The fiber type-specific expression of sarcolemmal FAT/CD36 and the colocalization of sarcolemmal FAT/CD36 with caveolin-3 prompted us to investigate whether the same fiber type-specific pattern of sarcolemmal expression existed for caveolin-3. As described above for FAT/CD36, we evaluated the fiber type-specific distribution of caveolin-3 on serial transverse cryosections from vastus lateralis muscle from five subjects labeled with either anti-caveolin-3 or anti-MHC-1 antibody. In fact, we observed that caveolin-3 expression in sarcolemma was distributed in a fiber type-specific pattern. However, surprisingly, cave-

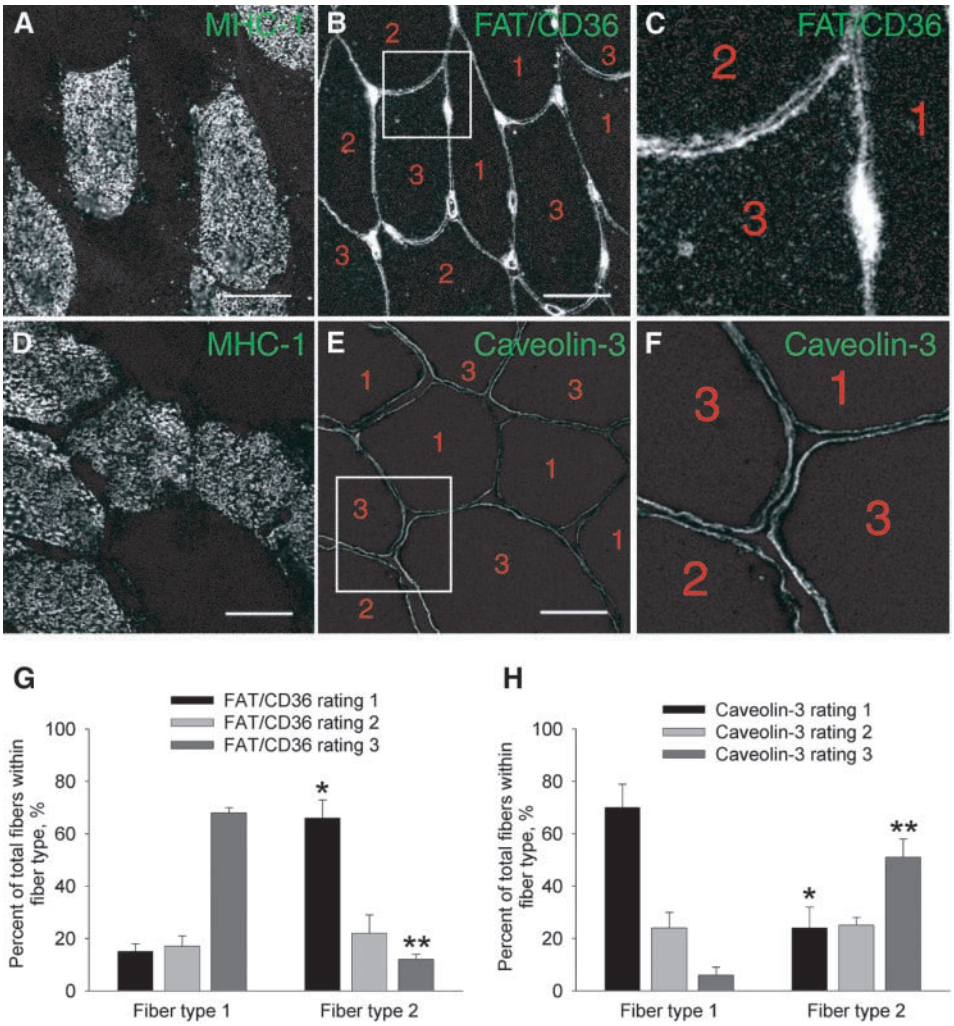

Fig. 4. Fiber type-specific distribution of sarcolemmal FAT/CD36 and caveolin-3. Serial transverse cryosections from human vastus lateralis muscle were fixed and stained with anti-MHC-1 mouse monoclonal antibody (A and D), anti-FAT/CD36 mouse monoclonal antibody (B and C), or anti-caveolin-3 mouse monoclonal antibody (E and F). The intensity of the fluorescent signal from sarcolemmal FAT/CD36 and caveolin-3 was rated in ~100 fibers in each subject. The percentage of type 1 and type 2 fibers given each rating was calculated (G and H) (n = 4–5). A and B: Two serial cryosections stained for MHC-1 and FAT/CD36 and their sarcolemmal FAT/CD36 ratings. In A, stained fibers are type 1 and other fibers are type 2. C: Magnification of the boxed area in B. D and E: Two serial cryosections stained for MHC-1 and caveolin-3 and their sarcolemmal caveolin-3 ratings. In D, stained fibers are type 1 and other fibers are type 2. F: Magnification of the boxed area in E. G and H: Diagrams showing the percentage of fibers within each fiber type rated 1, 2, or 3 on sarcolemmal FAT/CD36 or caveolin-3 intensity. * $P < 0.01$, ** $P < 0.001$ versus the same rating in fiber type 1. Bars = 30 μm in A, B, D, and E. Error bars are mean ± SE.

olin-3 was more abundantly expressed in sarcolemma of type 2 fibers than of type 1 fibers, i.e., the opposite pattern compared with FAT/CD36 (Fig. 4D–F, H).

DISCUSSION

It is well known that FAT/CD36 is expressed in human as well as rodent skeletal muscle (3, 4). However, the localization of this lipid binding protein in human muscle has not been clarified. The present study is the first to investigate by morphological techniques the distribution and subcellular localization of FAT/CD36 in human skeletal muscle. Four important and interesting findings have been demonstrated in human skeletal muscle tissue: *1*) FAT/CD36 is expressed primarily in endothelial cells and to a lesser degree in muscle cells; *2*) in muscle cells, FAT/CD36 is associated with the sarcolemma; *3*) the distributions of FAT/CD36 and caveolin-3 in the sarcolemma are similar; and *4*) the expression of FAT/CD36 is higher in type 1 than in type 2 fibers, whereas the expression of caveolin-3 is higher in type 2 than in type 1 fibers.

From the present findings in human skeletal muscle tissue, it is obvious that FAT/CD36 is expressed to a larger extent in endothelial cells than in the sarcolemma, supporting previous morphological evidence in rats (19).

Skeletal muscle is an important site for fatty acid uptake, especially during exercise, in which the rate of fatty acid uptake into skeletal muscle can increase manyfold compared with the resting state (20). For long-chain fatty acids to enter the parenchymal cells from the blood stream, the layer of endothelial cells surrounding the capillaries is the first barrier to pass. Therefore, FAT/CD36 expressed by endothelial cells in muscle tissue may function as transporters of fatty acids across the endothelium (21).

It is clear from the present findings using confocal immunofluorescence microscopy that FAT/CD36 in human skeletal muscle is associated with the sarcolemma and that no staining of FAT/CD36 was evident in intracellular compartments. These findings in human skeletal muscle are supported by our own observations (data not shown) and those of others in rat skeletal muscle (19). On the other hand, from fractionation studies of homogenates obtained from rat skeletal and heart muscle, it was shown that FAT/CD36 is present in both an intracellular pool and in the plasma membrane (7, 8, 22, 23). Those studies also demonstrated that with muscle contractions and insulin stimulation, part of the intracellular pool of FAT/CD36 was translocated to the plasma membrane (7, 8, 22, 23). The glucose transporter GLUT4 is well recognized to translocate from intracellularly located tubular-vesicular structures to the plasma membrane by regulated exocytosis (15, 16), a process that shares several characteristics with the mechanism proposed for the translocation of FAT/CD36 (24). However, GLUT4 has clearly been demonstrated by immunohistochemistry to reside in intracellular tubular-vesicular compartments (16, 17), in agreement with our own findings demonstrating intracellular staining for GLUT4 (Fig. 2A). Furthermore, we have found by Western blotting on cytosolic and total crude membrane (TCM) fractions obtained from human skeletal muscle that FAT/CD36 is localized exclusively to membrane structures (TCM) in the muscle cell (data not shown). This suggests that FAT/CD36 does not merely have an even distribution throughout the cytosol. Thus, if translocation of FAT/CD36 from an intracellular compartment to the sarcolemma occurs, one might expect a picture similar to that of GLUT4 localization, but this was not the case. However, because of the optical resolution of the microscope, we were not able to detect very small tubular-vesicular structures, so the possibility exists that a very small amount of FAT/CD36 is bound to intracellular membrane structures. Furthermore, it cannot be ruled out that part of the FAT/CD36 protein detected as sarcolemmal in fact localizes to compartments immediately beneath the sarcolemma, as the optical resolution of the microscope cannot discern between FAT/CD36 localized to the sarcolemma and FAT/CD36 localized to a subsarcolemmal compartment. However, we are not aware of any distinct subsarcolemmal compartments in skeletal muscle resembling the distribution of FAT/CD36 shown in Figs. 2C and 3D, G.

A number of recent studies in mammalian cell lines and caveolin-1 knockout mice have suggested that caveolae or their marker protein, caveolin, are involved in the cellular uptake of fatty acids (9, 11, 25). There are several putative mechanisms whereby caveolae/caveolin may regulate cellular fatty acid uptake. First, caveolin-1 in adipocytes was shown to be a fatty acid binding protein itself (25). It has also been suggested that caveolae are involved in endocytosis of fatty acids (26). Alternatively, plasma membrane caveolae may regulate the function of fatty acid transporters such as FAT/CD36. Therefore, we found it interesting to investigate the localization of FAT/CD36 relative to the muscle-specific isoform of caveolin, caveolin-3, in human skeletal muscle, a tissue in which fatty acid uptake rates can at times be immense (20). As previously shown in rat skeletal muscle (17) and in cultured muscle cells (27), we found that sarcolemmal caveolin-3 showed a distinct, striated pattern (Fig. 3E, H, K). Interestingly, FAT/CD36 at the surface of the fiber shows the same striated pattern and seems to colocalize with caveolin-3 (Fig. 3F, I). Although the resolution of a light microscope does not allow the detection of single caveolae, the very similar distribution of FAT/CD36 and caveolin-3 in the sarcolemma indicates that FAT/CD36 may indeed localize to caveolae in human skeletal muscle. To our knowledge, this is the first study to suggest that FAT/CD36 is located in caveolae in skeletal muscle. Interestingly, by sucrose density gradient centrifugation, FAT/CD36 in lung tissue has previously been shown to localize to low-density membrane domains such as caveolae or noncaveolar lipid rafts (28). Furthermore, a recent study has shown that immunopurified caveolae from adipocytes contain FAT/CD36 (29). Further studies are needed to investigate the possible roles of caveolae in the function of FAT/CD36 and other fatty acid transporters.

In the present study, we demonstrated by morphology that in human skeletal muscle the expression of FAT/CD36 is higher in type 1 than in type 2 muscle fibers. Recently, similar findings were obtained in rat skeletal muscle, in which morphological studies revealed that muscle fibers in soleus muscle have a higher expression of FAT/CD36 than do muscle fibers in the white gastrocnemius muscle (19). Western blotting has shown that the amount of FAT/CD36 measured in giant sarcolemmal vesicles obtained from rat skeletal muscle was 40% higher in red than in white muscles (3). The fiber type-specific pattern of expression of FAT/CD36 suits the putative role of FAT/CD36 in fatty acid transport from the interstitium to the muscle cell across the sarcolemma. Thus, because type 1 fibers have a higher fat oxidative potential compared with type 2 fibers (30), their need for fatty acid uptake is correspondingly higher.

Interestingly, in contrast to the fiber type distribution of FAT/CD36, caveolin-3 was expressed to a higher degree in type 2 fibers compared with type 1 fibers. This is the first time that the localization of caveolin-3 related to fiber types has been investigated in human skeletal muscle. The high expression of caveolin-3 in type 2 compared with type 1 fibers may appear surprising in the context of fatty acid transport. However, caveolae are thought to be involved in a plethora of cellular processes in addition to fatty acid transport, such as signaling from a variety of re-

ceptors and the regulation of the dystrophin-glycoprotein complex in skeletal muscle (10, 11). Therefore, the level of expression of caveolin-3 in muscle fibers may not only reflect a role in fatty acid uptake.

In summary, our data suggest a localization of FAT/CD36 in sarcolemma and endothelial cells of human skeletal muscle. Furthermore, we have indicated a colocalization of FAT/CD36 and caveolin-3 in sarcolemma. The expression of FAT/CD36 is fiber type-specific, with a significantly higher abundance in type 1 compared with type 2 fibers, whereas the expression of caveolin-3 is significantly higher in type 2 fibers compared with type 1 fibers.

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