Importance of the carboxyl terminus of FAT/CD36 for plasma membrane localization and function in long-chain fatty acid uptake[S]

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Abstract This study investigates the role of the cytoplasmic C terminus of fatty acid translocase (FAT/CD36) in localization of the molecule to the plasma membrane, its insertion into lipid rafts, and its ability to enhance long-chain fatty acid uptake in transfected H4IIE rat hepatoma cells. In these cells, wild-type FAT/CD36 is localized to both lipid raft and nonraft domains of the plasma membrane. Interestingly, a FAT/CD36 truncation mutant lacking the final 10 amino acids of the cytoplasmic C terminus was retained within the cell in detergent-resistant membranes, and unlike wild-type FAT/CD36, it did not enhance oleate uptake. Furthermore, expression of FAT/CD36 in these cells increased the incorporation of oleate into diacylglycerol, a property that was not shared by truncated FAT/CD36. To examine whether the C terminus itself has an intrinsic ability to dictate the plasma membrane localization of FAT/CD36, this region was fused in-frame to enhanced green fluorescent protein (EGFP). This domain was sufficient to attach EGFP to cellular membranes, suggesting an involvement in the intracellular traffic of the molecule. We conclude that the C terminus of FAT/CD36 is required for localization of the receptor to the cell surface and its ability to enhance cellular oleate uptake.—Eyre, N. S., L. G. Cleland, N. N. Tandon, and G. Mayrhofer. **Importance of the carboxyl terminus of FAT/CD36 for plasma membrane localization and function in long-chain fatty acid uptake. *J. Lipid Res.* 2007.** 48: **528–542.**

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Fatty acid translocase (FAT; or CD36) is an ~88 kDa glycoprotein that mediates the uptake of oxidized low density lipoprotein (OxLDL) by macrophages and is an important mediator of long-chain fatty acid (LCFA) uptake in muscle and adipose tissue (reviewed in Ref. 1). The generation and study of FAT/CD36 gene knockout mice and transgenic mice with muscle-specific overexpression of FAT/CD36 has demonstrated the importance of this molecule in fatty acid transport and metabolism (2, 3). Although LCFAs can diffuse across cell membranes (4), active transport via FAT/CD36 is particularly important in tissues with high metabolic demand for LCFAs, especially when plasma free fatty acid levels are low (2, 5). Factors involved in regulating FAT/CD36 localization and activity are the subject of intense investigation.

Like other class B scavenger receptors, such as scavenger receptor class B type I, FAT/CD36 is anchored in the plasma membrane by transmembrane domains at N and C termini and contains a large extracellular loop that is highly *N*-glycosylated (6–8). Human FAT/CD36 is palmitoylated at membrane-proximal cysteine residues in the N- and C-terminal cytoplasmic tails, and it is thought that this lipidation may serve to target and/or strengthen the attachment of the molecule to the plasma membrane (7). Furthermore, because palmitoylation is reversible, it may regulate the translocation of FAT/CD36 between intracellular sites and the plasma membrane. However, palmitoylation at the C-terminal end of the molecule is unnecessary for its localization to the cell surface or to confer enhanced binding and uptake of OxLDL on transfected HEK293 cells (9).

Nevertheless, the C terminus of FAT/CD36 is important functionally. Deletion of the C-terminal lysine residue results in reduced binding and uptake of OxLDL, and this effect is exacerbated when the final six amino acids are deleted (9). Furthermore, the $CXCX_5K$ motif in this re-

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Abbreviations: CT-B, cholera toxin B subunit; DGAT, diacylglycerol acyltransferase; DPBS, Dulbecco's phosphate-buffered saline; DRM, detergent-resistant membrane; EGFP, enhanced green fluorescent protein; FAT, fatty acid translocase; LCFA, long-chain fatty acid; OxLDL, oxidized low density lipoprotein.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of 1 figure.

gion of the molecule is a predicted site of interaction with tyrosine kinases (6).

At the subcellular level, FAT/CD36 is reported to localize to plasma membrane microdomains known as caveolae (10). Caveolae are 50–100 nm flask-shaped invaginations of the plasma membrane that are rich in cholesterol and sphingolipids and have been implicated in various aspects of lipid metabolism (reviewed in Ref. 11). Caveolin-1, the marker protein of caveolae, is a 22 kDa integral membrane protein whose expression and oligomerization are both necessary and sufficient to induce the formation of morphological caveolae (12, 13). Interestingly, FAT/CD36-mediated LCFA uptake and lipid raft function have been linked in a recent study involving 3T3-L1 adipocytes (14). That study showed that the effects of blocking FAT/CD36-mediated LCFA uptake with sulfo-*N*-succinimidyl-oleate and of limiting lipid raft-dependent LCFA uptake by depletion of cholesterol with cyclodextrin were nonadditive. With respect to the purported caveolar localization of FAT/CD36, Zeng and colleagues (15) found only minimal colocalization of FAT/CD36 and caveolin-1 in transfected CHO cells, although both molecules were enriched in detergent-resistant lipid rafts. This finding suggests that FAT/CD36 can also reside in lipid rafts that are distinct from caveolae and that the proportions of FAT/CD36 localized to caveolae and to noncaveolar lipid rafts may differ between cell types. Significantly, we have shown that FAT/CD36 is expressed in hepatocytes (16), although its subcellular localization and primary function in these cells remain uncertain.

In the work described here, we investigated the localization of FAT/CD36 to lipid rafts/caveolae in transfected H4IIE rat hepatoma cells and rat liver. Furthermore, we studied the influence of FAT/CD36 expression on LCFA uptake and metabolism in transfected H4IIE cells and the effects of truncation of the C terminus of the molecule on these properties and its subcellular localization. Finally, we investigated the propensity of the cytoplasmic C terminus of FAT/CD36 to influence the attachment of the molecule to membranes, including lipid raft-derived detergent-resistant membranes (DRMs).

METHODS

Cells, antibodies, and other ligands

H4IIE rat hepatoma cells, COS-7 cells, and HEK293 cells were grown in DMEM supplemented with 10% FBS, 2 mM glutamine, 10 μg/ml streptomycin, and 10 U/ml penicillin in a 5% CO_2/95% air atmosphere at 37°C. Mouse monoclonal anti-FAT/CD36 antibodies UA009 and MO25 have been described previously (16, 17), as has the mouse monoclonal anti-transferrin receptor antibody OX-26 (18). The following antibodies were purchased: mouse monoclonal anti-caveolin-1 (clone C060; BD Transduction Laboratories, Lexington, KY), biotinylated goat polyclonal anti-green fluorescent protein (Rockland Immunochemicals, Gilbertsville, PA), mouse monoclonal anti-β-actin (Sigma, St. Louis, MO), horseradish peroxidase-conjugated anti-mouse IgG $F(ab')_2$ (Amersham Biosciences, Piscataway, NJ), FITC-conjugated anti-mouse immunoglobulin (BD Pharmingen, San Diego, CA), and Cy3-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Horseradish peroxidase-conjugated streptavidin was purchased from Amersham, and Alexa Fluor 594 cholera toxin B subunit (CT-B) conjugate was purchased from Molecular Probes (Eugene, OR).

DNA constructs and transfection

Rat FAT/CD36 cDNA was subcloned from pSG5-CD36 (19) (a generous gift from Dr. Nada Abumrad) into pcDNA3 (Invitrogen, Carlsbad, CA) using *Eco*RI and *Xba*I restriction sites (pcDNA3-FAT/CD36). To generate a caveolin-1-enhanced green fluorescent protein (EGFP) fusion construct (pCav1-EGFP), rat caveolin-1 cDNA was amplified by PCR from Dark Agouti rat liver cDNA using the primers 5′-CCGAAGCTTATGTCTGGGGGTAAATACGTAGAC-3′ and 5′-GGATCCCG*TAT*CTCTTCCTGCGTGCTGATG-3′. It was then subcloned in-frame into pEGFP-N1 (Clontech Laboratories, Palo Alto, CA) using *Hind*III and *Bam*HI restriction sites (underlined). The mutated stop codon is italicized. To generate a caveolin-1-EGFP fusion construct featuring a puromycin resistance cassette, the entire caveolin-1-EGFP coding region was subcloned from pCav1-EGFP into pEF-IRES-puro6 (a generous gift from Dr. Daniel Peet) using *Xho*I and *Not*I restriction sites (pEF-Cav1-EGFP-IRES-puro6). To generate a FAT/CD36 construct lacking the final 5 or 10 amino acid residues, FAT/CD36 was first subcloned from pcDNA3-FAT/CD36 into pcDNA6/V5-His A (Invitrogen) as a *Bam*HI fragment to give pcDNA6/V5-His A-FAT/CD36. FAT/CD36 cDNA fragments of ~550 bp were subsequently amplified by PCR using pSG5-CD36 as template DNA, 5′-TGTTCTTCCAGCCAACGCCT-3′ as the forward primer, and one of the following reverse primers, 5′-AGACTCGAGCG*CTA*TCTGCAAGCACAGTATGAAATC-3′ (FAT/CD36del5) or 5′-AGACTCGAGCGCTATCTGCAAGCACA*CTA*TGAAATC-3′ (FAT/CD36del10) (*Xho*I restriction sites are underlined, and stop codons that were introduced are italicized). PCR products were digested with *Pst*I and *Xho*I, and fragments were subcloned into *Pst*I and *Xho*I sites of pcDNA6/V5-HisA-FAT/CD36. To generate a chimeric construct featuring the cytoplasmic C-terminal tail of FAT/CD36 fused to the C terminus of EGFP (pEGFP-Cterm-FAT/CD36), the following oligonucleotides (encoding the final 14 amino acid residues and featuring *Hind*III and *Bam*HI overhangs) were annealed and ligated into the *Hind*III and *Bam*HI sites of pEGFP-C1 (Clontech): 5′-AGCTTCTGGATTTATGATTTCATACTGTGCTTGCAGATCTAAGAATGGAAAATAAG-3′ and 5′-GATCCTTATTTTCCATTCTTAGATCTGCAAGCACAGTATGAAATCATAAATCCAGA-3′. To generate a chimeric construct containing the previously described membrane attachment sequence (KYWFYR) of caveolin-1 (20) fused to the C terminus of EGFP (pEGFP-CavKYWFYR), the following oligonucleotides encoding the KYWFYR peptide and also containing *Hind*III and *Bam*HI overhangs were annealed and ligated into the *Hind*III and *Bam*HI sites of pEGFP-C1: 5′-AGCTTCTGGAAAATATTGGTTTTACCGCTAGG-3′ and 5′-GATCCCTAGCGGTAAAACCAATATTTTCCAGA-3′. All constructs were confirmed by automated DNA sequencing using Dye Terminator Chemistry and a 3700 DNA Sequencer (Applied Biosystems, Foster City, CA).

To generate H4IIE cell lines stably expressing FAT/CD36, cells were transfected with pcDNA3-FAT/CD36 using FuGene6 (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. Stable transfectants were selected by the addition of G418 (Life Technologies, Burlington, Ontario, Canada), and clonal transfectants were isolated, expanded, and screened for FAT/CD36 expression by flow cytometry. One clone, named H4IIE-FAT/CD36 (1A), demonstrated high expression of FAT/CD36 and was chosen for further analy-

sis. Another clone, named H4IIE (5A), demonstrated no expression of FAT/CD36 and was chosen as a negative control. Each of these clones was stably transfected with pEF-Cav1-EGFP-IRES-puro6 as described above, selecting with puromycin (Sigma). To generate stable cell lines expressing each of the mutant FAT/CD36 molecules, H4IIE cells were transfected with pcDNA6/V5-HisA-FAT/CD36del5 or pcDNA6/V5-HisA-FAT/CD36del10 as described above, selecting stable transfectants with blasticidin (Sigma). These cell lines, named H4IIE-FAT/CD36del5 and H4IIE-FAT/CD36del10, were transfected with pCav1-EGFP as described above using G418 to select for stable transfectants. To generate stable cell lines expressing EGFP (H4IIE-EGFP), the C terminus of FAT/CD36 fused to EGFP (H4IIE-EGFP-Cterm-FAT/CD36), or the KYWFYR membrane attachment sequence of caveolin-1 fused to EGFP (H4IIE-EGFP-KYWFYR), H4IIE cells were transfected with pEGFP-C1, pEGFP-Cterm-FAT/CD36, or pEGFP-CavKYWFYR, and stable transfectants were selected with G418. Where applicable, EGFP-positive cells were enriched by fluorescence-activated cell sorting using a FACSAria cell sorter (Becton Dickinson, San Jose, CA).

Transient transfections of COS-7 cells were performed according to the manufacturer's instructions. Briefly, cells were seeded at 2×10^5 cells per well in six-well trays or at 4.5×10^5 cells per 60 mm dish. Cells were cultured overnight and transfected using a FuGene6:DNA ratio of 3:1 (μl/μg). Assays were performed 48 h after transfection.

Cell surface biotinylation and streptavidin precipitation

Cell surface biotinylation was performed essentially as described (21). Briefly, cells were grown to confluence in 60 mm cell culture dishes, washed three times with ice-cold PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ (PBS-C/M), and then incubated with 1 ml of sulfo-NHS-biotin (Pierce Biotechnologies, Rockford, IL) (0.5 mg/ml in PBS-C/M) for 20 min at 4°C with gentle agitation. This buffer was discharged, and fresh biotinylation agent was added for another 20 min. Cell monolayers were then washed once with ice-cold DMEM and three times with ice-cold PBS-C/M, before lysis on ice in TNE buffer [25 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 5 mM EDTA] containing 1% Triton X-100 plus appropriately diluted protease inhibitor cocktail (Sigma). Postnuclear supernatants were prepared by centrifugation. The protein concentration in each lysate was determined by the Bradford procedure (Bio-Rad Laboratories, Hercules, CA). For each streptavidin precipitation, 500 μg of cellular protein, adjusted to 0.5 mg/ml, was mixed with 20 μl of streptavidin-conjugated agarose beads (Sigma) and incubated at 4°C by end-over-end rotation for 2 h. The beads were washed four times with lysis buffer before heating at 95°C for 5 min in 50 μl of 2× SDS-PAGE loading buffer [250 mM Tris (pH 6.8), 4% SDS, 10% glycerol, 2% β-mercaptoethanol, and 0.006% bromophenol blue]. The streptavidin precipitates, together with 20 μg of cellular protein from each sample, were subjected to 12% SDS-PAGE and immunoblotting.

Confocal fluorescence microscopy

Cells were grown on sterile coverslips overnight before labeling for immunofluorescence. Where applicable, they were cultured for 1 h at 37°C in the presence of 0.5 μg/ml Alexa Fluor 594-CT-B in DMEM containing 5% FBS. The coverslips were washed with PBS, fixed for 6 min on ice using 5% buffered formalin in Dulbecco's phosphate-buffered saline (DPBS), and rinsed with DPBS containing 1% FBS. For indirect immunofluorescence, the cells were labeled with monoclonal antibody UA009 (neat culture supernatant containing 10% heat-inactivated normal rat serum) for 1 h at 4°C. After washing, bound antibody was detected by incubation for 1 h in the dark at 4°C with FITC-conjugated or Cy3-conjugated anti-mouse Ig secondary antibodies (1:100 and 1:150, respectively, in DPBS containing 10% normal rat serum). For the detection of intracellular antigens in fixed cells, saponin (0.1%, w/v) was included in wash buffers and antibody preparations. After three washes, coverslips were mounted on glass slides with Vectashield mounting medium containing 4′,6-diamidino-phenylindole (Vector Laboratories, Burlingame, CA), and the cells were visualized and photographed using a Bio-Rad MRC-1000UV confocal laser scanning microscope system and a Nikon Diaphot 300 inverted microscope (see Figs. 1C, 4 below). Alternatively, samples were visualized and photographed using a Leica SP5 spectral scanning confocal microscope.

Preparation of DRMs

DRMs were prepared essentially as described by Peng et al. (22), with minor variations. Briefly, cells were grown to confluence in 100 mm tissue culture dishes, washed twice with PBS, and lysed on ice in 0.7 ml of TNE buffer containing 1% Triton X-100 and protease inhibitors. Alternatively, small pieces of liver from female DA strain rats (aged 7–8 weeks) were snap-frozen in liquid nitrogen, crushed to a fine powder using a liquid nitrogen-cooled mortar and pestle, and stored at −80°C. Approximately 50 mg of liver powder was solubilized in TNE buffer containing 1% Triton X-100 and protease inhibitors. In each case, lysates were transferred to microcentrifuge tubes, passed through a 26 gauge needle 10 times, and incubated on ice for 30 min. Samples were then centrifuged at 1,000 *g* to remove nuclear debris, and 0.5 ml of the cleared lysate was transferred to a new tube and mixed with an equal volume of 80% (w/v) sucrose in TNE buffer. The mixture was then transferred to a 4.5 ml SW60 centrifuge tube, and the sample was overlaid with 2.5 ml of 38% (w/v) sucrose and 1 ml of 5% (w/v) sucrose in TNE buffer. After centrifugation at 38,000 rpm (148,305 *g*) for 15 h at 4°C, 12 fractions were collected from the top of the tube. Equal volumes of each fraction were subjected to SDS-PAGE and immunoblotting.

Cell surface biotinylation and streptavidin precipitation of DRMs

As described above, confluent cell monolayers in 100 mm cell culture dishes were surface-biotinylated using sulfo-NHS-biotin (Pierce). Biotinylated cells were then lysed in 0.7 ml of TNE buffer containing 1% Triton X-100, and the postnuclear lysates were subjected to sucrose gradient fractionation. DRM fractions (fractions 2–4) were pooled, as were fractions referred to collectively as "non-raft" fractions (fractions 8–12). Protein in the DRM fractions was below the limits of detection by the Bradford procedure. These fractions (1 ml total), therefore, were used undiluted for the precipitation of biotinylated proteins using streptavidin-conjugated agarose (as described above). In the case of the nonraft fraction, 500 μg of protein was diluted in TNE buffer containing 1% Triton X-100 to a final volume of 1 ml, and this was used for streptavidin precipitation of nonraft transmembrane proteins. Streptavidin precipitates and 20 μl of the poststreptavidin supernatant ("wash") were subjected to 12% SDS-PAGE and immunoblotting.

Hypotonic lysis

Fractionation of cellular proteins into soluble and particulate fractions by hypotonic lysis and high-speed centrifugation was performed essentially as described (20). Briefly, cells were grown to confluence in 60 mm cell culture dishes and washed twice with PBS before scraping into 5 ml of ice-cold PBS. Cells were then centrifuged, and the pellets were resuspended in 0.5 ml of cold hypotonic lysis buffer containing protease inhibitor cocktail (Sigma). After incubation on ice for 30 min, samples were passed through

a 26 gauge needle 10 times and centrifuged at 1,000 *g* for 5 min. Postnuclear supernatants were transferred to polycarbonate tubes (11 × 34 mm) and centrifuged at 41,000 rpm (~112,000 *g*) for 30 min in a TLS-55 rotor (Beckman) at 4°C. Supernatants were collected, and after gently rinsing with 200 μl of cold hypotonic lysis buffer, the pellets were resuspended in an equal volume of 1% SDS. Equal volumes of soluble and particulate fractions were subjected to SDS-PAGE and immunoblotting.

Extraction of Triton-soluble proteins

Triton extraction was performed essentially as described (20). Briefly, confluent cell monolayers grown in six-well cell culture trays were washed twice with ice-cold PBS before the addition of 350 μl of cold MBS [25 mM MES (pH 6.5) and 150 mM NaCl] containing 1% Triton X-100 and protease inhibitors. After incubation on ice for 30 min, soluble fractions were collected. The residual monolayers were then rinsed gently with cold MBS before the addition of 350 μl of 1% SDS to collect the Triton X-100-insoluble fractions. Equal volumes of Triton X-100-soluble and -insoluble fractions were subjected to SDS-PAGE and immunoblotting.

Immunoblotting

Protein samples were subjected to 12% SDS-PAGE under reducing conditions and transferred to Hybond-P polyvinylidene difluoride membranes (Amersham). Membranes were blocked for 1 h with 7.5% BSA in T-TBS (TBS containing 0.1% Tween-20) before incubating overnight with primary antibody (MO25 at 1:2,000, biotinylated anti-GFP at 1:5,000, anti-caveolin-1 at 1:500, OX-26 hybridoma supernatant at 1:10, or anti-β-actin at 1:10,000) diluted in T-TBS containing 0.5% BSA. Membranes were washed thoroughly with T-TBS and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (anti-mouse-HRP at 1:20,000) or streptavidin-HRP (1:20,000) diluted in T-TBS containing 0.5% BSA. Membranes were then washed thoroughly with T-TBS before detection using SuperSignal West Femto (Pierce). Where applicable, bands were quantified using a high-resolution scanner and NIH Image software.

Assay of oleate uptake

Assays were performed essentially as described (23), with minor modifications. Oleic acid solution was prepared by mixing trace amounts of [^{14}C]oleic acid (~5 μCi; Amersham) with nonradioactive oleic acid (Sigma) and dissolving in a fatty acid-free BSA (Sigma) solution (173 μM) in PBS to achieve an oleic acid/BSA molar ratio of 1. Cells were seeded at a density of 3×10^5 cells per well in 12-well tissue culture trays. After overnight culture, they were washed twice with warm PBS before addition of 300 μl of warm oleic acid solution. After incubation at 37°C for the appropriate times, the oleic acid solution was replaced with 1 ml of ice-cold stop solution containing 200 μM phloretin (Sigma) and 0.5% BSA in PBS. After 1 min, the stop solution was aspirated and the cells were washed twice (1 min each) with ice-cold PBS containing 0.5% BSA and once with ice-cold PBS and then lysed in 250 μl of NaOH (2 mol/l). Aliquots of the NaOH lysate were used for protein determination by the Bradford procedure and measurement of radioactivity. To measure radionuclide, 200 μl of lysate was added to 1.5 ml of OptiPhase HiSafe 3 liquid scintillation cocktail (Perkin-Elmer Life Sciences, Boston, MA), and samples were analyzed using a Wallac 1409 liquid scintillation counter (Wallac Oy).

Cellular lipid separation by TLC

Incorporation of [^{14}C]oleic acid into cellular lipids was determined after incubation of cells with oleic acid solution for 15 min, as described above. However, instead of lysing cells, lipids were extracted by two incubations with 0.3 ml of hexane-isopropanol (3:2). The extracts were pooled, the solvent was evaporated by vacuum centrifugation, and the lipids were redissolved in chloroform-methanol (2:1). Samples and standards, including oleic acid and a mix of monoglycerides, diglycerides, and triglycerides (Sigma), were spotted onto Silica Gel 60 plates (Merck, Darmstadt, Germany). The lipids were separated by TLC, using a hexane-diethyl ether-acetic acid (60:40:1) solvent system, and visualized after staining with iodine vapor. Lipid spots were identified and scraped into liquid scintillation cocktail for scintillation counting. Dry cell monolayers were dissolved in NaOH (2 mol/l) for protein determination by the Bradford procedure.

Statistical analyses

Where applicable, results are presented as means ± SD. The significance of differences between means was analyzed by the unpaired Student's *t*-test (two-tailed).

RESULTS

Effects of truncation of the C terminus on cell surface expression of FAT/CD36

H4IIE rat hepatoma cells were chosen for transfection because they do not express endogenous FAT/CD36, although rat hepatocytes can express high levels of FAT/CD36 at the cell surface in vivo (16) and expression of FAT/CD36 has been reported in human liver (24). Two truncation mutants were generated to address the hypothesis that the C terminus of FAT/CD36 is required for localization of the molecule to the cell surface (**Fig. 1A**), and these constructs and an expression plasmid containing the coding sequence of wild-type FAT/CD36 cDNA were used to stably transfect the cells.

Surface expression of FAT/CD36 and truncation mutants in the transfected H4IIE cells was assessed by cell surface biotinylation and streptavidin precipitation. Western analysis of the precipitates revealed that both wild-type FAT/CD36 and FAT/CD36del5 were expressed at the cell surface. In contrast, FAT/CD36del10 was not detectable in plasma membrane proteins precipitated from transfectants (Fig. 1B). However, FAT/CD36del10 was easily detected by Western analysis of whole cell lysates, indicating that the molecule is retained in the cytoplasm. Importantly, reprobing of membranes with streptavidin-HRP demonstrated that biotinylation and the diversity of streptavidin-precipitated proteins were similar for all transfectants (data not shown). It was noteworthy that the apparent molecular mass of FAT/CD36del10 (~80 kDa) was less than that of FAT/CD36del5 and wild-type FAT/CD36 (~85 kDa). Treatment of denatured lysates prepared from the respective H4IIE transfectants with peptide-N-glycosidase F (PNGase F) revealed that the mobilities of wild-type FAT/CD36 and the truncated proteins were indistinguishable after deglycosylation (see supplementary Fig. I), indicating that glycosylation of FAT/CD36del10 was incomplete relative to the wild-type protein.

Immunofluorescence confocal microscopy confirmed that the subcellular distribution of wild-type FAT/CD36 and FAT/CD36del5 was similar. In contrast, FAT/CD36del10 was detected in perinuclear organelles that may repre-

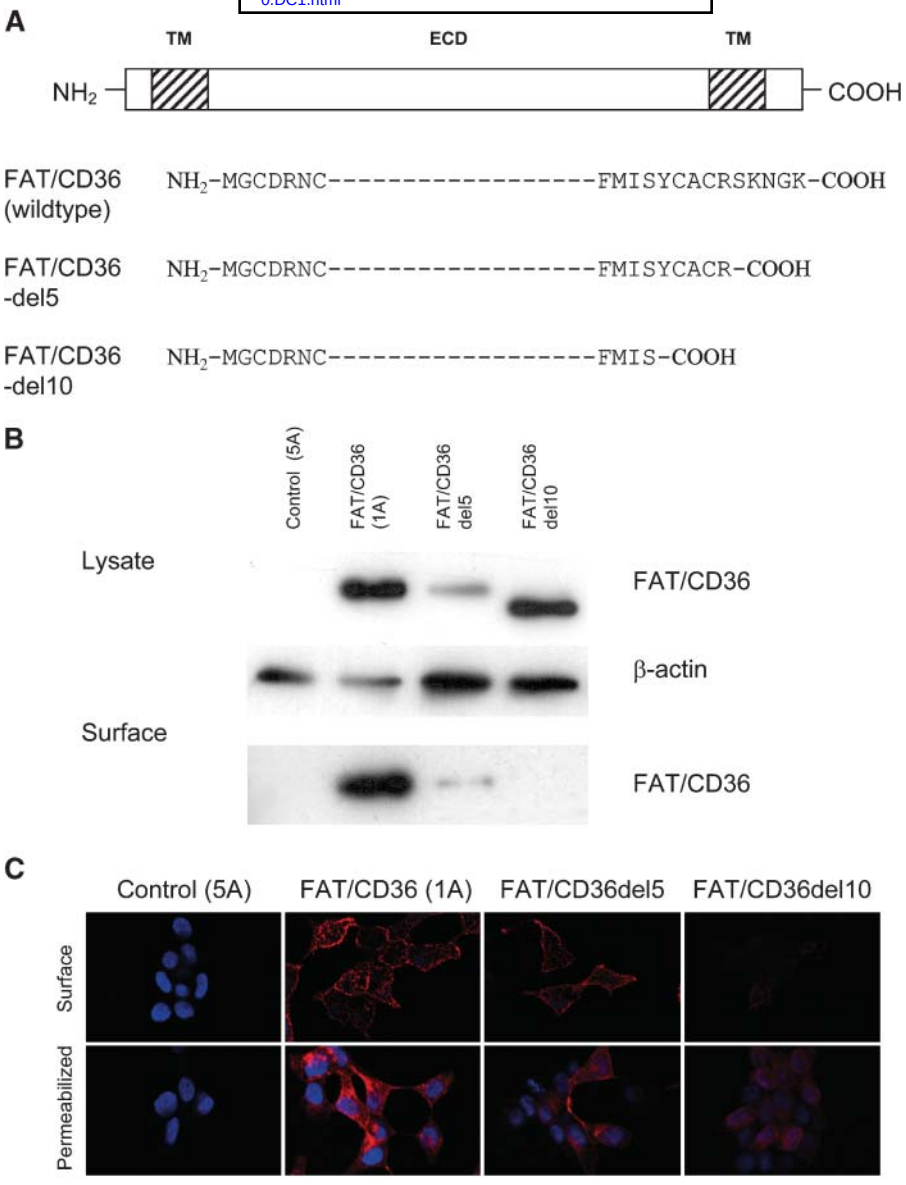

Fig. 1. Truncation mutants were used to explore the involvement of the cytoplasmic C-terminus in the intracellular trafficking of fatty acid translocase (FAT/CD36). A: Schematic diagrams of wild-type FAT/CD36 and C-terminal truncation mutants. Hydrophobic transmembrane domains (TM) are depicted as hatched boxes, which define the boundaries of the extracellular domain (ECD) spanning the region. B: Western blot analysis of surface-labeled proteins from transfectants expressing FAT/CD36 or truncation mutants FAT/CD36del5 and FAT/CD36del10. Cells were surface-biotinylated and lysed with 1% Triton X-100 in TNE buffer, and the biotinylated proteins were captured with streptavidin-conjugated agarose beads. Samples of lysates (20 μg) and streptavidin precipitates were subjected to SDS-PAGE and immunoblotting with monoclonal antibodies against FAT/CD36 (monoclonal antibody MO25) or β-actin. Results are representative of replicate experiments. C: Localization of FAT/CD36 and FAT/CD36 truncation mutants by confocal laser scanning microscopy (×40 objective plus ×3 zoom). Cells were grown on coverslips, fixed, and stained by indirect immunofluorescence (red) to detect FAT CD36 (monoclonal antibody UA009 plus Cy3-conjugated anti-mouse Ig) either with (intracellular) or without (cell surface) permeabilization. Nuclei were stained with 4′,6-diamino-phenylindole (blue).

sent endoplasmic reticulum, but it was not detected at the plasma membrane (Fig. 1C).

Localization of FAT/CD36 in DRMs

We have shown previously that FAT/CD36 is expressed by hepatocytes in rat liver in a gender-dependent manner (16). To examine whether FAT/CD36 is associated with lipid rafts in liver, 1% Triton X-100 extracts prepared from samples obtained from female DA strain rats were subjected to sucrose density gradient centrifugation to separate lipid raft-derived DRMs. Equal volumes of each fraction were analyzed by SDS-PAGE and immunoblotting (**Fig. 2A**). Most of

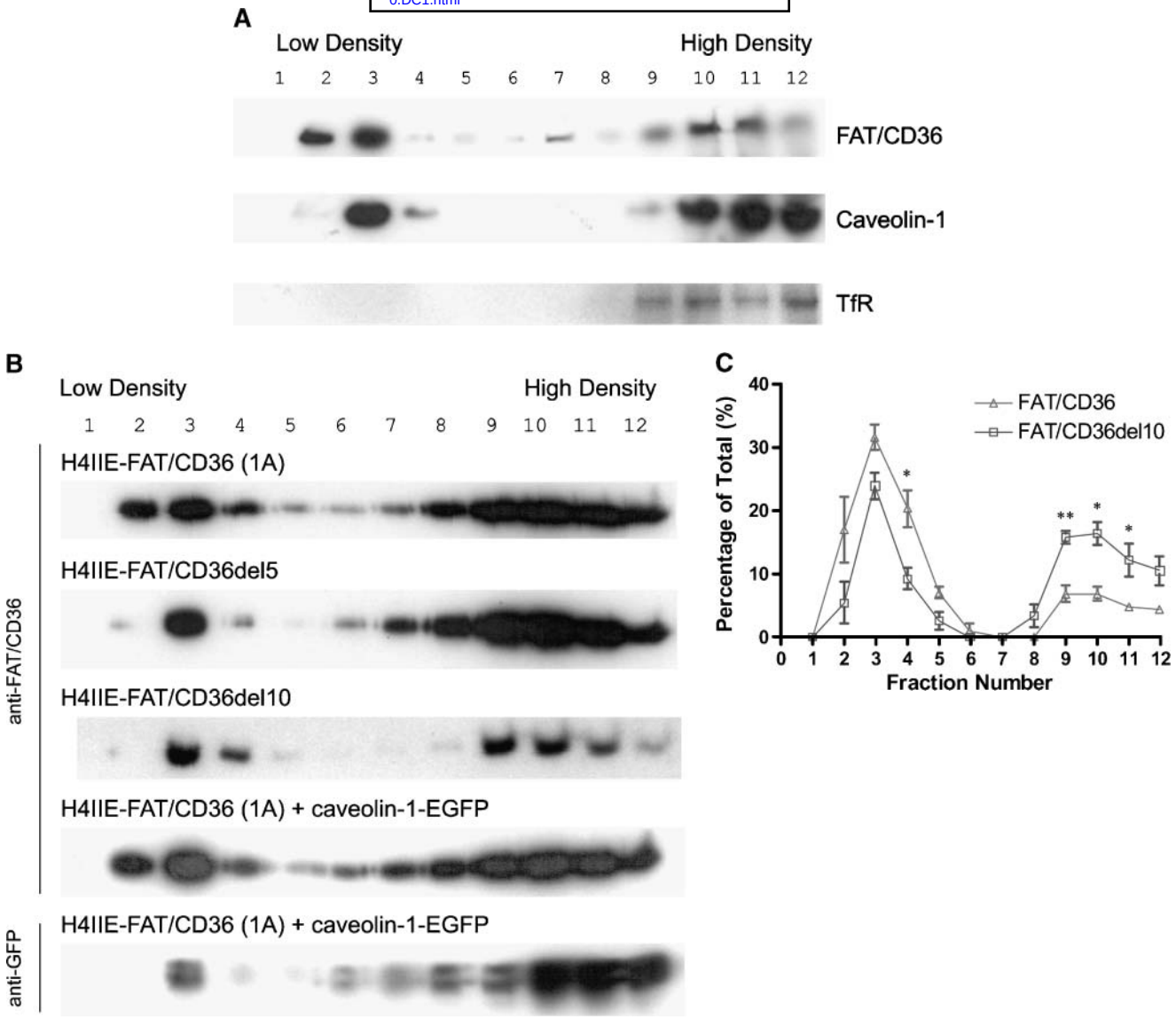

Fig. 2. Analysis of detergent-resistant membranes (DRMs) from rat liver and H4IIE hepatoma cells. A: Western blot analysis of FAT/CD36 in DRMs prepared from rat liver. Portions of liver (~50 mg) from female DA strain rats were homogenized in 1% Triton X-100 at 4°C, and the lysates were subjected to discontinuous 5–40% sucrose gradient centrifugation. Fractions were collected from the tops of the gradients, and aliquots from each were subjected to 12% SDS-PAGE and immunoblotting using anti-FAT/CD36 (monoclonal antibody MO25), anti-caveolin-1 (monoclonal antibody C060), or anti-transferrin receptor (TfR; monoclonal antibody OX-26) antibodies. Low-density DRMs contain both FAT/CD36 and caveolin-1. B: Western blot analysis of FAT/CD36 in DRM preparations from stably transfected H4IIE cells expressing wild-type FAT/CD36, FAT/CD36del5, or FAT/CD36del10. The association of FAT/CD36 and caveolin-1 with DRM fractions was also assessed in H4IIE cells stably transfected with wild-type FAT/CD36 and caveolin-1-enhanced green fluorescent protein (EGFP), as indicated. After lysis in 1% Triton X-100 at 4°C, samples were subjected to discontinuous 5–40% sucrose gradient centrifugation. Aliquots from each fraction were subjected to SDS-PAGE (12%) and immunoblotting using anti-FAT/CD36 or anti-GFP antibodies, as indicated. C: Measurement of FAT/CD36 content in fractions from sucrose gradients after centrifugation of Triton X-100 lysates prepared from stable transfectants expressing FAT/CD36 or FAT/CD36del10. The content of immunoreactive FAT/CD36 in each fraction was calculated from Western blots by densitometry and expressed as a percentage of total reactivity with anti-FAT/CD36 across all fractions. Values are means ± SD for three replicates. * $P < 0.05$, ** $P < 0.01$.

the immunoreactive FAT/CD36 was associated with the DRM-containing fractions, which also contained caveolin-1. In contrast, the transferrin receptor was excluded from DRMs, indicating that the DRM fractions were not contaminated with nonraft components. Thus, most of the FAT/CD36 in liver is associated with DRMs, where it cofractionates with caveolin-1.

To investigate the effects of truncation of the C terminus of FAT/CD36 on its insertion into lipid rafts, 1% Triton X-100 lysates from stably transfected H4IIE cell lines expressing the relevant constructs were subjected to sucrose step-gradient fractionation (25). Analysis of the fractions by SDS-PAGE and Western blotting shows that, as in liver, wild-type FAT/CD36 is enriched in DRMs in transfected H4IIE cells (Fig. 2B). Furthermore, truncation of the C terminus (FAT/CD36del5 and FAT/CD36del10) does not prevent incorporation of the molecule into DRMs. However, when the proportion of FAT/CD36 and FAT/

CD36del10 in DRMs was compared by densitometric analysis, it was found that the proportion of the wild-type protein that localized in the DRM-containing fractions was significantly greater than that of the FAT/CD36del10 mutant protein (Fig. 2C). Importantly, reprobing of membranes with an antibody against the transferrin receptor (a non-raft-associated membrane protein) showed that the DRM fractions were not contaminated with this nonraft protein (data not shown).

The enrichment of FAT/CD36 and FAT/CD36 truncation mutants in DRM fractions supports the localization to sphingolipid- and cholesterol-rich lipid rafts. These lipid rafts appear to be distinct from caveolae because H4IIE cells lack detectable expression of caveolin-1 (as determined by RT-PCR; data not shown). Caveolae are a subset of classical lipid rafts that are resistant to solubilization in 1% Triton X-100 at 4°C, and they are defined by morphology and enrichment with caveolin-1 (10). To examine whether caveolin-1 expression, and hence the formation of caveolae, influences the distribution of FAT/CD36, a vector encoding a caveolin-1-EGFP fusion protein was expressed stably in the H4IIE-FAT/CD36 (1A) cell line. Others have shown that caveolin-1-EGFP fusion proteins are indistinguishable from wild-type caveolin-1 in their localization, oligomerization, palmitoylation, and enrichment in DRMs (26–28). When expressed in H4IIE cells, the fusion protein was enriched in the DRM fraction (Fig. 2B). Furthermore, the presence of caveolin-1-EGFP had only a modest effect on the proportion of FAT/CD36 recovered in DRM from H4IIE-FAT/CD36 (1A) cells stably transfected with caveolin-1-EGFP (~52%) compared with H4IIE-FAT/CD36 (1A) cells alone (~44%). Thus, the presence of caveolin is not necessary for the incorporation of FAT/CD36 into DRM in cells of this rat hepatoma.

Localization of FAT/CD36 and deletion mutants to caveolae and lipid rafts, as determined by confocal fluorescence microscopy

As noted above, FAT/CD36 cofractionated with caveolin-1-EGFP in DRMs prepared from cotransfected cells. However, FAT/CD36 was also detected in DRMs prepared from transfected H4IIE cells that lack caveolin-1. These

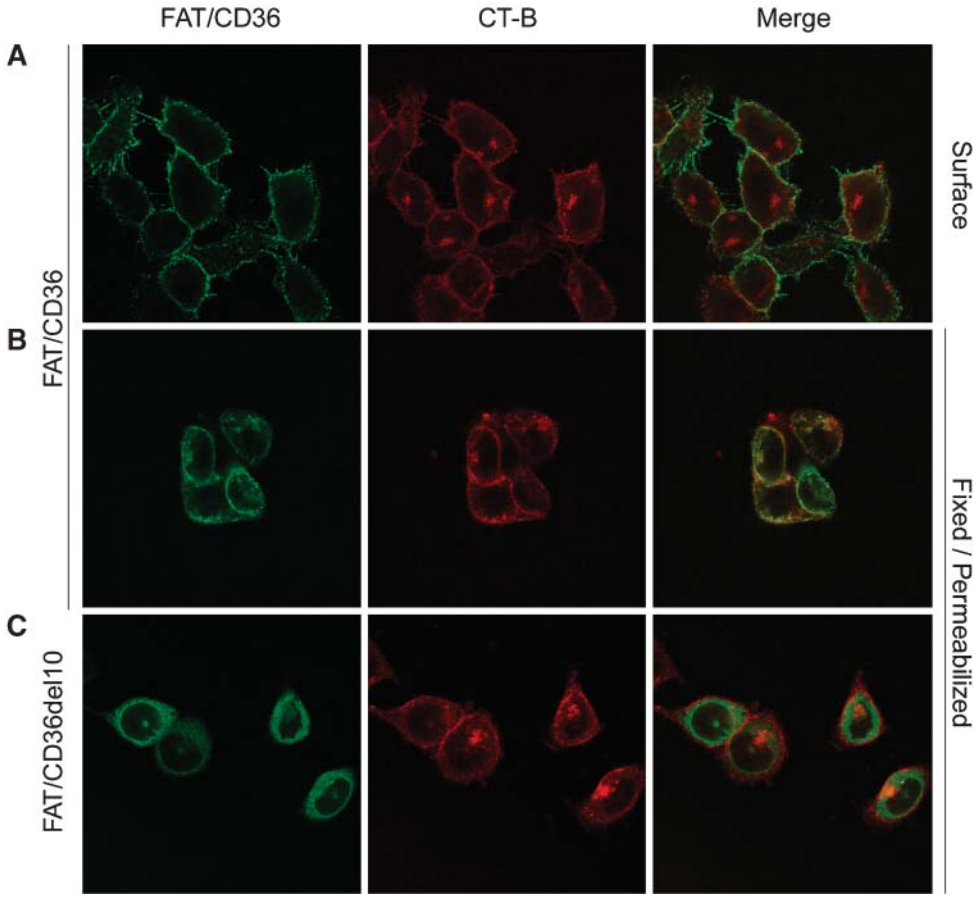

Fig. 3. Colocalization of FAT/CD36 and lipid rafts by confocal fluorescence microscopy. Stable H4IIE transfectants expressing either wild-type FAT/CD36 (A and B) or FAT/CD36del10 (C) were grown on coverslips. Washed monolayers were incubated at 37°C with Alexa Fluor 594-conjugated cholera toxin B subunit (CT-B) for 1 h to allow binding and uptake (red). Cells were fixed (A) or fixed and permeabilized with 0.1% saponin (B and C). They were then labeled by indirect immunofluorescence (green) to detect FAT/CD36 (monoclonal antibody UA009 plus FITC-conjugated anti-mouse Ig). Images were made with a ×63 objective plus a ×3 zoom.

observations indicate that FAT/CD36 can reside in lipid raft domains that are distinct from caveolae. The subcellular localization of FAT/CD36 was examined by colabeling cells with Alexa Fluor 594-conjugated CT-B, to define lipid raft domains, and with monoclonal antibody UA009, to detect FAT/CD36. FAT/CD36 was found to colocalize with bound CT-B, both at the cell surface and within cells (**Fig. 3**, upper and middle panels). It appears, therefore, that FAT/CD36 is associated with GM_1-enriched lipid rafts and that some of these rafts are intracellular. The subcellular distribution of FAT/CD36del5 and its association with CT-B were indistinguishable from those of wild-type FAT/CD36 (data not shown). However, as shown in Fig. 1, FAT/CD36del10 was detectable only after permeabilization of the H4IIE transfectants, where it was localized in cytoplasmic organelles that overlapped partially with CT-B bound to cytoplasmic Golgi-like structures (Fig. 3).

Although the results from sucrose density gradient centrifugation analysis indicate some colocalization of FAT/CD36 with caveolin-1, this technique does not separate caveolae from sphingolipid- and cholesterol-rich lipid rafts that lack caveolin-1 (29, 30). Confocal fluorescence microscopy was used to examine whether FAT/CD36 colocalizes to the same membrane domains as caveolin-1 when H4IIE-FAT/CD36 (1A) cells are transfected with caveolin-1-EGFP. As seen in **Fig. 4**, wild-type FAT/CD36 and caveolin-1-EGFP were partially colocalized in the plasma membranes of the cotransfected cells. Furthermore, after fixation and permeabilization before labeling, colocalization of the molecules was observed also within large perinuclear Golgi-like organelles in the cytoplasm. The distribution of FAT/CD36del5 and its spatial association with caveolin-1-EGFP were similar. In contrast, FAT/CD36del10 was localized to intracellular organelles that shared little overlap with caveolin-1-EGFP (data not shown).

Distribution of FAT/CD36 in lipid raft and nonraft components of the plasma membrane

To investigate whether cell surface FAT/CD36 is associated with lipid rafts exclusively (14), transfected H4IIE-FAT/CD36 (1A) cells were surface-labeled with membrane-impermeable biotin before preparation of DRMs. Consistent with the findings shown in Fig. 2, FAT/CD36 was readily detectable in DRMs and also in the detergent-soluble fractions (cytosolic proteins and detergent-soluble membrane proteins) (**Fig. 5A**). Reprobing with streptavidin-HRP verified that surface biotinylation had been successful and indicated that very few transmembrane proteins were associated with DRMs in these cells. To examine whether FAT/CD36 in the DRM fractions and the detergent-soluble fractions is among the surface biotinylated proteins, the pooled fractions were precipitated separately with streptavidin-agarose. Biotinylated FAT/CD36 was precipitated from both fractions, indicating that the FAT/CD36 at the cell surface is associated with both detergent-soluble membranes and DRMs (Fig. 5B). Smaller amounts of FAT/CD36 remained in both fractions after the streptavidin-agarose precipitation, and most of this material was removed by a second treatment with streptavidin-agarose (data not shown). Reprobing the membrane with streptavidin-HRP revealed a biotinylated doublet in the DRM fraction, the upper band having a molecular mass similar to that of FAT/CD36 (Fig. 5A, B).

Membrane attachment of FAT/CD36 and truncation mutants

FAT/CD36, FAT/CD36del5, and FAT/CD36del10 were all associated with DRMs in transfected H4IIE cells, despite cytoplasmic retention of FAT/CD36del10. However, it was unclear whether the FAT/CD36 that was associated with nonraft fractions was associated with membranes and, in the case of FAT/CD36del10, whether defective membrane association contributed to intracellular retention.

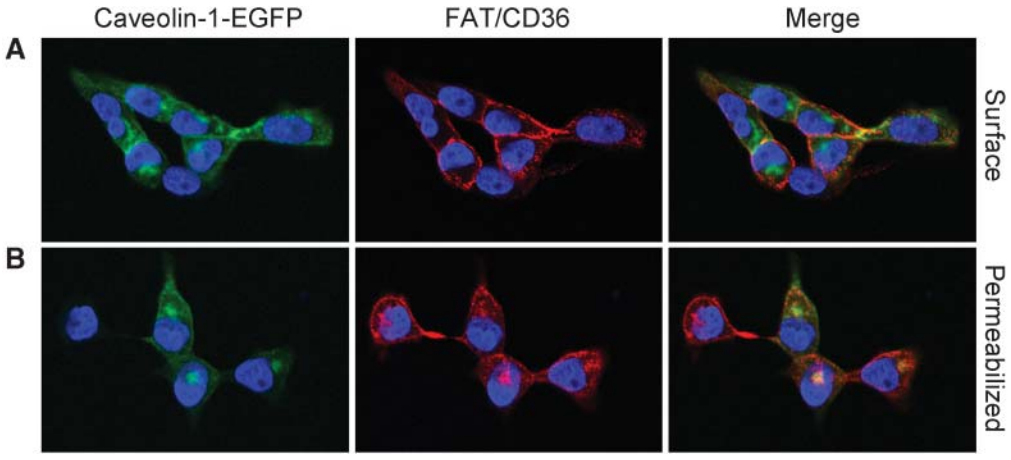

Fig. 4. Colocalization of FAT/CD36 and caveolin-1-EGFP in H4IIE hepatoma cells. Cells were grown on coverslips and either fixed (A) or fixed and permeabilized (B) before staining by indirect immunofluorescence (red) to detect FAT/CD36 (monoclonal antibody UA009 plus Cy3-conjugated anti-mouse Ig). Nuclei were stained with 4′,6-diamino-phenylindole (blue). Images were made with a ×40 objective plus a ×3 zoom.

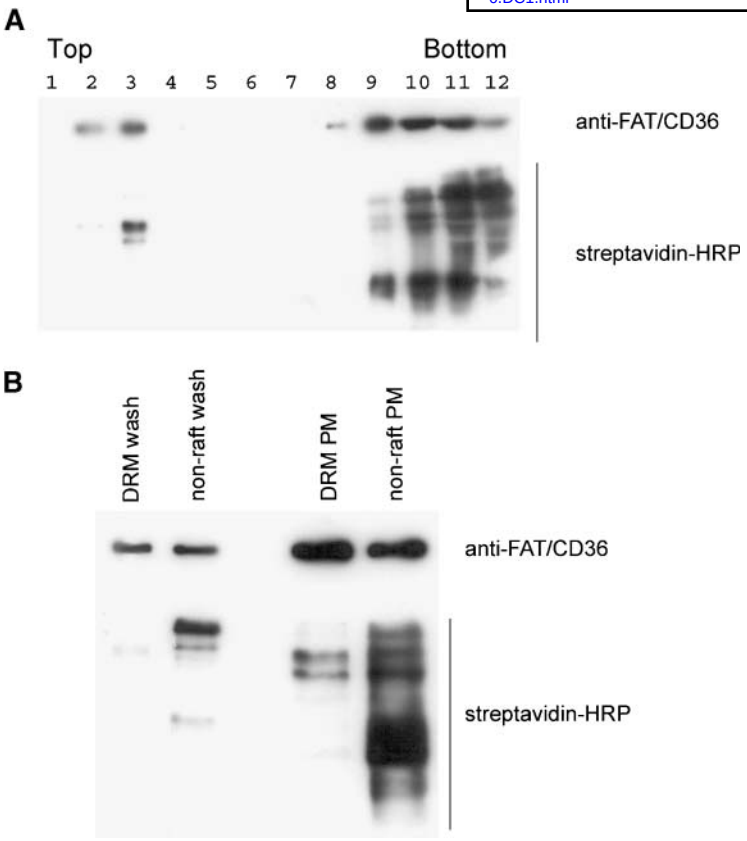

Fig. 5. Enrichment of FAT/CD36 in lipid raft and nonraft components of the plasma membrane. H4IIE cells stably expressing FAT/CD36 were surface-biotinylated, lysed in 1% Triton X-100 at 4°C, and subjected to discontinuous 5–40% sucrose gradient centrifugation as detailed in Methods. A: Aliquots from each fraction were subjected to SDS-PAGE and immunoblotting with anti-FAT/CD36 using monoclonal antibody MO25 (upper panel). Immunoblots were then stripped and reprobed with streptavidin-HRP (lower panel). B: Fractions 2–4 were pooled (DRM fractions), as were fractions 8–12 (nonraft fractions), and biotinylated plasma membrane (PM) proteins were precipitated from each using streptavidin-conjugated agarose beads. Streptavidin precipitates and 20 μl from the washings were subjected to SDS-PAGE and immunoblotting with anti-FAT/CD36 (upper panel). Membranes were then stripped and reprobed with streptavidin-HRP (lower panel).

To examine this, cellular proteins were fractionated into soluble and particulate fractions by hypotonic lysis and ultracentrifugation. Both wild-type FAT/CD36 and the truncated FAT/CD36 molecules were found in each case to be associated predominantly with membrane fractions (**Fig. 6A**). To further dissect any effects of C-terminal truncation on the partitioning of FAT/CD36 into membranes, the solubility of native FAT/CD36 and the truncation mutants in 1% Triton X-100 at 4°C was assessed as detailed in Methods. The Triton X-100-soluble and -insoluble proteins were then analyzed by Western blot. As shown in Fig. 6B, wild-type and truncated forms of FAT/CD36 were all associated mainly with the Triton X-100-insoluble fractions. Together, these results demonstrate that the wild-type and truncated FAT/CD36 proteins (including FAT/CD36del10) are predominantly membrane-bound.

The C terminus of FAT/CD36 facilitates membrane attachment

Retention of the truncated FAT/CD36del10 protein within the cytoplasm suggests the presence of a plasma membrane targeting motif in the deleted region. To investigate

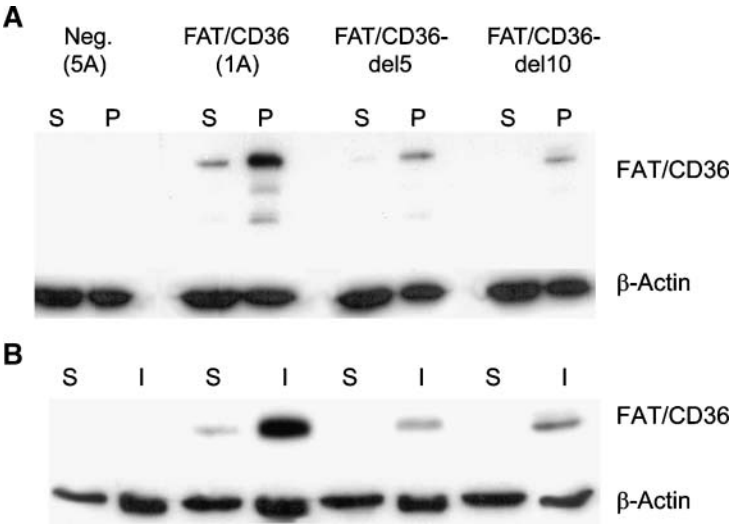

Fig. 6. Membrane association of wild-type FAT/CD36 and C terminus truncation mutants. A: H4IIE cells stably expressing wild-type FAT/CD36, FAT/CD36del5, or FAT/CD36del10 were subjected to hypotonic lysis, and the postnuclear supernatants were ultracentrifuged to separate soluble proteins (S) from particulate membrane-bound proteins (P). Equal volumes from each fraction were subjected to SDS-PAGE and immunoblotting to detect FAT/CD36 (monoclonal antibody MO25) and β-actin. B: Alternatively, confluent cell monolayers were incubated with 1% Triton X-100 in MBS [25 mM MES (pH 6.5) and 150 mM NaCl] at 4°C to release detergent-soluble proteins (S). Residual Triton X-100-insoluble proteins (I) were then solubilized in an equal volume of 1% SDS. Equal volumes from each fraction were then subjected to SDS-PAGE and immunoblotting. Results shown are typical of replicate experiments.

this possibility, a construct encoding the terminal 14 amino acid residues appended to the coding sequence of the soluble reporter EGFP was transfected into H4IIE and COS-7 cells. The distribution of the chimeric protein in the transfectants was compared in membranes prepared by hypotonic lysis (**Fig. 7A**) and in Triton X-100-soluble and -insoluble fractions prepared from transfectants (Fig. 7B). Cells transfected with a construct encoding the minimal membrane attachment sequence of caveolin-1 (KYWFYR) appended to EGFP (20) were included as a positive control. Inclusion of the C terminus of FAT/CD36 was sufficient to associate a significant proportion of the EGFP fusion protein with membranes in H4IIE and COS-7 cells (Fig. 7A). As expected, no EGFP was found in association with membranes prepared

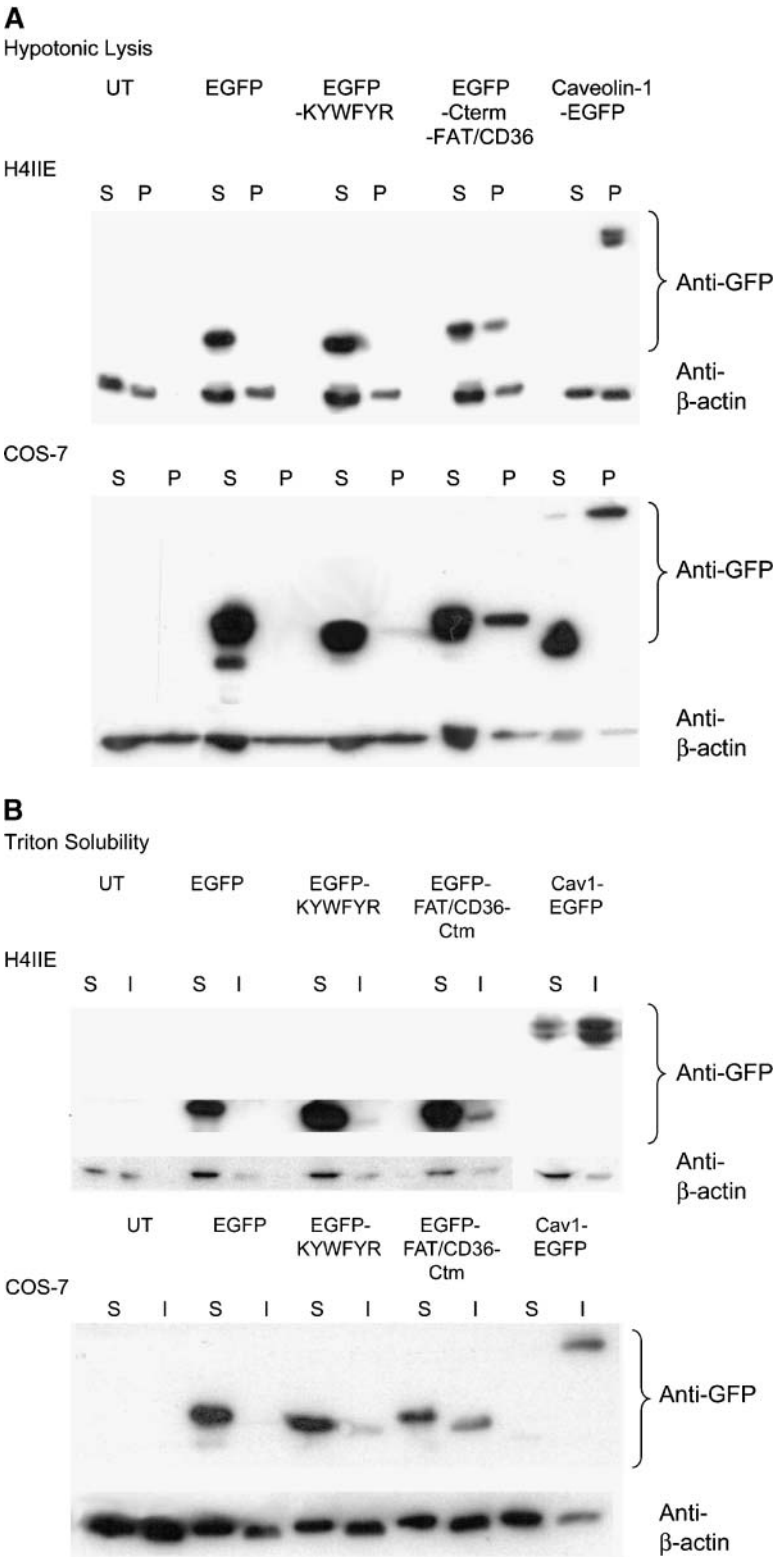

Fig. 7. The C terminus of FAT/CD36 confers membrane association to EGFP. A: Stably transfected H4IIE cells (upper panels) and transiently transfected COS-7 cells (lower panels) were subjected to hypotonic lysis, and the membrane fraction was pelleted by ultracentrifugation. Transfectants expressed the EGFP fusion protein containing the terminal 14 amino acids of FAT/CD36 (EGFP-C-term-FAT/CD36), EGFP, EGFP-KYWFYR, or caveolin-1-EGFP. Untransfected cells (UT) were used as controls. Equal volumes from the soluble (S) and particulate (P) fractions were subjected to SDS-PAGE and immunoblotted with the antibodies indicated. B: The detergent solubility of the fusion proteins in the above transfectants was assessed by lysis of the cells with 1% Triton X-100. Samples from the soluble (S) and insoluble (I) fractions were subjected to SDS-PAGE and immunoblotting. Results shown are typical of replicate experiments.

from cells expressing unmodified EGFP, whereas the caveolin-1-EGFP chimeric protein partitioned almost exclusively with the membrane fractions. Unexpectedly, EGFP-KYWFYR was not detected in membranes prepared from H4IIE transfectants and was only detected in trace amounts in membranes prepared from transfected COS-7 cells. Although much of the EGFP fusion protein containing the C terminus of FAT/CD36 was associated with the detergent-soluble fraction prepared from transfected cells, a significant amount was associated also with the detergent-insoluble fraction, as was most of the caveolin-1-EGFP fusion protein (Fig. 7B). In contrast, EGFP was present in the detergent-soluble fraction exclusively, and only traces of the EGFP-KYWFYR chimeric protein were not detergent-soluble.

To investigate whether the C terminus of FAT/CD36 facilitates the association of EGFP with DRMs, H4IIE transfectants were lysed in 1% Triton X-100 at 4°C and the extract was fractionated by sucrose step gradient ultracentrifugation. Although unmodified EGFP and KYWFYR-EGFP were detected only in fractions that are expected to contain soluble proteins and nonraft membrane proteins (fractions 9–12), EGFP bearing the C terminus of FAT/CD36 was detected in the DRM fraction, albeit at low levels (**Fig. 8**). As a positive control, H4IIE cells stably expressing full-length caveolin-1-EGFP were subjected to the same treatment in parallel. As expected, this chimeric protein was enriched in the DRM fraction.

Effects of a cytoplasmic C-terminal truncation of FAT/CD36 on LCFA uptake and incorporation into cellular lipids

To assess the contribution of the C terminus (either directly or through its effects on the subcellular localization of FAT/CD36) to high-affinity LCFA uptake, cells expressing wild-type FAT/CD36, FAT/CD36del5, or FAT/CD36del10 were incubated with [^{14}C]oleic acid adsorbed to equimolar concentrations of fatty acid-free BSA (see Methods). The concentration of LCFA and the ratio of fatty acid to BSA were within accepted physiological ranges (31). Uptake was essentially linear in the H4IIE (5A) (control) and H4IIE-FAT/CD36 (1A) cell lines, and oleate uptake was increased significantly in cells expressing FAT/CD36 (**Fig. 9A**). Expression of either FAT/CD36 or FAT/CD36del5 enhanced uptake significantly at 5 min, whereas expression of FAT/CD36del10 was accompanied by a significant reduction in uptake relative to H4IIE (5A) cells (Fig. 9B).

TLC separation of lipids revealed that expression of FAT/CD36 in H4IIE cells resulted in a 2-fold increase in the incorporation of radiolabeled oleate into diacylglycerol (**Table 1**). However, this increase was not seen in cells expressing either of the truncated molecules. Furthermore, overexpression of FAT/CD36 caused a significant decrease in oleate incorporation into triacylglycerol. This effect was mirrored in cells expressing FAT/CD36del5, whereas expression of FAT/CD36del10 did not affect oleate incorporation into triacylglycerol.

DISCUSSION

It is clear from studies in cardiac and skeletal muscle cells that there are both surface and intracellular stores of FAT/CD36 and that there can be a rapid interchange in response to stimuli such as contraction and application of

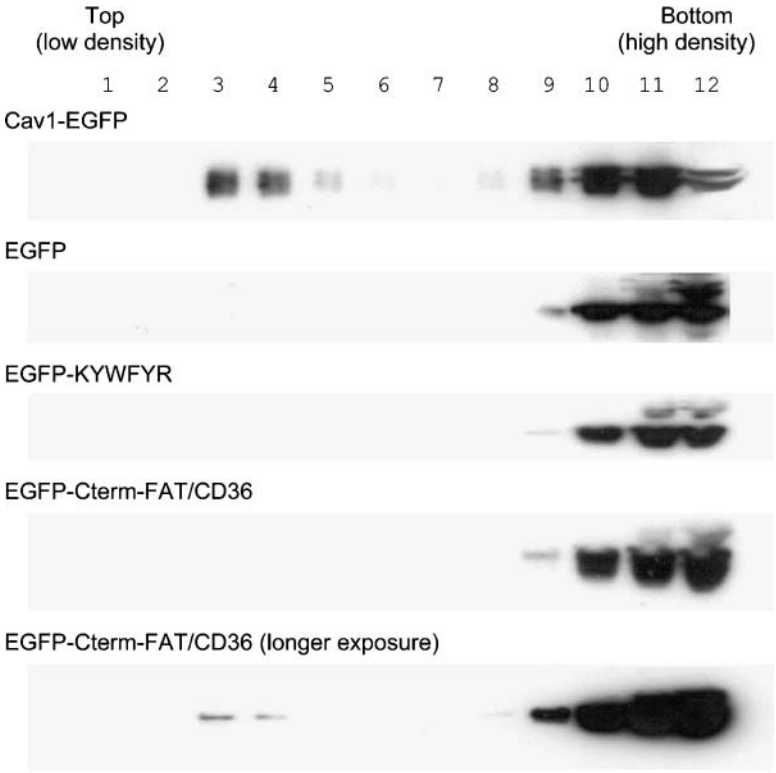

Fig. 8. Association of EGFP chimeric proteins with detergent-resistant membranes (DRMs) in transfected H4IIE hepatoma cells. H4IIE cells stably expressing caveolin-1-EGFP, EGFP, EGFP-KYWFYR, or the EGFP fusion protein containing the terminal 14 amino acids of FAT/CD36 (EGFP-C-term-FAT/CD36) were lysed in 1% Triton X-100 at 4°C, and the lysates were subjected to discontinuous 5–40% sucrose gradient centrifugation. Equal volumes from the fractions were subjected to SDS-PAGE and immunoblotted with an anti-GFP antibody. Representative Western blots are shown.

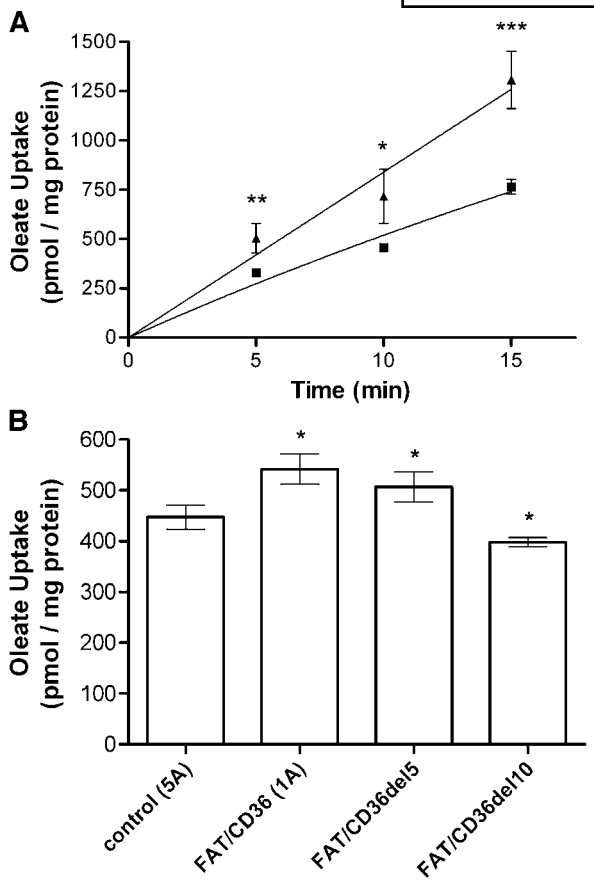

Fig. 9. Uptake of [^{14}C]oleic acid by stably transfected H4IIE hepatoma cells expressing either wild-type FAT/CD36 or C terminus truncation mutants. A: Oleate uptake at 37°C by H4IIE (5A) and H4IIE-FAT/CD36 (1A) cell lines (squares and triangles, respectively). Cells were incubated with [^{14}C]oleate solution (173 μM) plus equimolar BSA for the times indicated. Fatty acid uptake was stopped by replacing the radioactive oleate solution with ice-cold PBS containing 0.5% BSA and 200 μM phloretin. Cell monolayers were then washed and processed for measurement of radioactivity and protein content as detailed in Methods. Values are means ± SD for four replicates at each time point. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$ (significant differences in uptake compared with control cells). Results are typical of three replicate experiments. B: Comparison of oleate uptake by H4IIE (5A), H4IIE-FAT/CD36 (1A), H4IIE-FAT/CD36del5, and H4IIE-FAT/CD36del10 cell lines after incubation with [^{14}C]oleate (173 μM solution with equimolar BSA) for 5 min. Values are means ± SD for six replicates. Results are typical of three replicate experiments. * $P < 0.005$ (significant difference compared with control cells).

insulin (32–34). In these cells, mobilization of FAT/CD36 to the cell surface provides a rapid response to the needs of the cell for energy production from LCFAs. It is not known whether similar cytoplasmic stores exist in other cell types, but nevertheless, there must be traffic of newly synthesized FAT/CD36 molecules from the cytoplasm to the plasma membrane. Recent studies in mouse embryonic fibroblasts have suggested that caveolin-1 is required for the expression of FAT/CD36 at the plasma membrane (35), whereas localization of FAT/CD36 to caveolae may be necessary for the molecule to function in LCFA transport (14, 35). We recently showed that FAT/CD36 is expressed by hepatocytes in rat liver (16). In this study, we have investigated aspects of the subcellular localization of FAT/CD36 in a rat hepatocyte cell line and in normal rat liver, with special reference to the role of the C terminus in the intracellular traffic of the molecule and the association with lipid rafts and caveolae.

FAT/CD36 consists of a large highly glycosylated extracellular loop that is anchored to the plasma membrane via short cytoplasmic tails at the N and C termini (7, 8, 36). The N-terminal transmembrane domain of FAT/CD36 is necessary for its localization to the cell surface, as deletion of this region results in retention in Golgi-like organelles (8). We show here that FAT/CD36 lacking the C-terminal 10 amino acid residues (YCACRSKNGK) is retained in endoplasmic reticulum-like organelles. In contrast, there was no retention after deletion of the C-terminal five amino acids, suggesting that the YCACR motif is involved in membrane targeting of FAT/CD36. Alternatively, the YCACR motif could affect the conformation of neighboring regions that are required for plasma membrane localization or for interaction with an adaptor protein analogous to PDZK1, the adaptor protein of scavenger receptor class B type I (reviewed in Ref. 37).

Targeting of proteins to the plasma membrane and to lipid rafts appears to require at least two cooperative signals, such as fatty acylation plus the presence of a polybasic region (reviewed in Ref. 38). The membrane-proximal cysteine residues are palmitoylated at positions 464 and 466 in the C terminus of human FAT/CD36 (7, 8). However, mutation of these residues does not abrogate surface expression of the molecule in transfected HEK293 cells (9). Nevertheless, the importance of the C terminus is emphasized by our findings that truncation of the C-terminal 10 amino acids abrogates the association of FAT/CD36 with the plasma membrane in both H4IIE hepatoma cells (Fig. 1) and HEK293 kidney epithelial cells (see supplementary material).

In contrast, a recent study in transfected HEK293 cells demonstrated that alanine substitutions at all positions in the cytoplasmic C terminus did not prevent the surface expression of FAT/CD36 (39). The reasons for the difference between these findings and our own in HEK293 cells suggest that it is the simple presence or absence of a cytoplasmic segment that is important. However, the results of our experiments using an EGFP fusion protein reporter containing the C terminus of FAT/CD36 do not support this suggestion. Data from these experiments support a role for the C terminus in the attachment of FAT/CD36 to membranes, because overexpression in H4IIE cells led to the recovery of a significant proportion of the fusion protein in membrane fractions, including DRM fractions. However, the presence of a large proportion of the fusion protein in the nonmembrane fractions of hypotonic lysates suggests that the interactions responsible for nonintegral attachment to membranes are relatively weak or that close proximity of the EGFP tag exerts an inhibitory effect on binding. Alternatively, palmitoylation of the chimeric protein might be suboptimal. Further studies are required to elucidate the nonintegral mechanisms that facilitate the attachment of FAT/CD36 to cellular mem-

TABLE 1. Effects of the expression of wild-type FAT/CD36 and FAT/CD36 truncation mutants on the incorporation of [^{14}C]oleic acid into complex cell lipids

Cell Line	Fatty Acid	Diglyceride	Triglyceride	Polar Lipids
Control (5A)	111.9 ± 21.3	867.6 ± 42.9	2,400.1 ± 156.4	9,442.6 ± 962.2
FAT/CD36 (1A)	116.0 ± 12.9	1,847.1 ± 178.5[a]	1,524.5 ± 89.9[a]	10,897.5 ± 1,060.5
FAT/CD36del5	146.9 ± 44.2	671.7 ± 62.11[b]	1,636.8 ± 161.0[a]	9,128.0 ± 701.8
FAT/CD36del10	149.1 ± 55.2	797.4 ± 86.6	2,921.5 ± 281.5	9,077.1 ± 852.7

FAT, fatty acid translocase. Polar lipids include phospholipids, phosphatidic acid, and monoacylglycerol 3-phosphate. Values are expressed as picomoles oleic acid incorporated/mg protein/15 min. Values are means ± SD for four replicates. Results are typical of three repeat experiments.
[a] $P < 0.0005$.
[b] $P < 0.005$.

branes, including lipid rafts. Ultimately, the C terminus is essential for the expression of FAT/CD36 at the plasma membrane, perhaps by ensuring passage of the molecule through the endoplasmic reticulum quality control processes (40) and/or transit through the *trans*-Golgi network for the completion of glycosylation. Our observation that the truncated FAT/CD36del10 protein is underglycosylated and retained in the cytoplasm in transfected H4IIE cells supports this general hypothesis.

An important feature of FAT/CD36 at the plasma membrane in adipocytes and muscle cells is its association with caveolae (10, 41–44). Association with lipid rafts/caveolae is thought to be important for the function of FAT/CD36 in LCFA transport. Depletion of lipid rafts by treatment with the cholesterol-sequestering agents filipin and β-cyclodextrin has been shown to reduce the uptake of LCFA by 3T3 adipocytes (45). Furthermore, caveolin-1 has been shown to bind fatty acids (46), and this had led to the hypothesis that FAT/CD36 and caveolin-1 may act in series to facilitate LCFA uptake (47). In this model, FAT/CD36 would transport or facilitate the diffusion of LCFAs across the plasma membrane, whereas the function of caveolin-1 would be to bind LCFAs or acyl-CoA derivatives at the inner leaflet and either present them to intracellular transporters or participate in their transport within the cell.

Our findings in transfected H4IIE hepatoma cells indicate that the expression of FAT/CD36 at the plasma membrane is not dependent on caveolin-1 and, therefore, the formation of caveolae. Furthermore, FAT/CD36 was localized to both detergent-resistant and detergent-soluble fractions of the plasma membrane in H4IIE cells (Fig. 5). Direct evidence that FAT/CD36 is associated with lipid rafts was obtained by colocalization studies with CT-B binding to GM_1 glycolipid. Interestingly, although retained in perinuclear organelles, the FAT/CD36del10 truncation mutant was also enriched in DRMs. These results allow us to conclude that FAT/CD36 partitions into lipid rafts within internal membrane compartments and that its localization to the plasma membrane requires the C terminus of the molecule. The colocalization of FAT/CD36 with the caveolin-1-EGFP fusion protein in cotransfected H4IIE cells suggests that FAT/CD36 can redistribute from classical lipid rafts to caveolae or that sphingolipid- and cholesterol-rich rafts can become caveolae as a result of enrichment with caveolin oligomers.

Despite the association of FAT/CD36 with caveolae in cotransfected cells, the expression of caveolin-1-EGFP did not increase the initial rates of either FAT/CD36-dependent or FAT/CD36-independent LCFA uptake in H4IIE hepatoma cells (data not shown). While this article was in preparation, it was reported that LCFA uptake is compromised in embryonic fibroblasts derived from caveolin-1 knockout mice (35). This phenotype was attributable to intracellular retention of FAT/CD36 in these caveolin-1-deficient cells. Therefore, the results in fibroblasts are in contrast with our findings in hepatoma cells. They suggest that the dependence on caveolin-1 expression for the expression of FAT/CD36 at the plasma membrane and for activity of the molecule in LCFA transport may be cell type-specific. Such differences, if they exist, could explain why an association between FAT/CD36 and caveolae has not been observed in all cells (15, 41, 42). It is interesting that in rat liver most of the FAT/CD36 is associated with DRMs, where some (but not all) cofractionate with caveolin-1. On the other hand, FAT/CD36 is associated with DRMs in transfected H4IIE hepatoma cells, which do not express caveolin-1. Thus, at least in hepatocytes and hepatocyte-derived cells, there is not a perfect correlation between the expression of caveolin-1 and the localization of FAT/CD36 to caveolae, or between the presence of caveolin-1 and either the surface expression of FAT/CD36 or FAT/CD36-mediated LCFA uptake.

The expression of native FAT/CD36 in H4IIE hepatoma cells resulted in a small but significant increase in the initial rate of LCFA uptake, comparable to that in published studies in other cell types (19, 48, 49). In contrast, the FAT/CD36del10 truncation mutant, which is retained in cytoplasmic membranes, did not enhance LCFA uptake. A similar requirement for the surface expression of FAT/CD36 can be deduced from studies that correlated increases in LCFA uptake with translocation of the molecule from intracellular stores to the sarcolemma in heart and skeletal muscle (reviewed in Ref. 50). Although the function of FAT/CD36 in the liver is unknown, these findings suggest that, as in other cells, the molecule may augment the uptake of LCFAs by hepatocytes.

Furthermore, our results suggest that FAT/CD36 may influence the intracellular fate of LCFAs in hepatocytes. H4IIE cells expressing FAT/CD36 contained ~2-fold more radiolabeled diacylglycerol after uptake of [^{14}C]oleic acid compared with control cells. This difference was accompanied by a commensurate decrease in radiolabeled triacylglycerol. This result contrasts with other studies in which the overexpression of FAT/CD36 in myocytes was associated

with greater incorporation of LCFAs into triacylglycerol (48, 51). In hepatocytes, the rate of triacylglycerol synthesis is limited by fatty acid supply and the activity of the enzyme diacylglycerol acyltransferase (DGAT) (52, 53). These studies, and others (31), have suggested that the relatively low affinity of DGAT for acyl-CoA substrates serves to ensure that triacylglycerol synthesis takes place only when the needs for β-oxidation and phospholipid biosynthesis have been satisfied. Our results suggest that the expression of FAT/CD36 in hepatic cells affects the conversion of diacylglycerol to triacylglycerol. FAT/CD36 could, either directly or indirectly, channel newly acquired LCFAs toward phospholipid synthesis and/or β-oxidation pathways, thus limiting acyl-CoA levels and therefore limiting the supply of substrate to DGAT. The recent demonstration that mitochondrial membranes contain high levels of FAT/CD36, where it appears to participate in the mitochondrial import of LCFAs for oxidation, provides one mechanism by which the molecule could affect the routing of fatty acids within the cell (54, 55). However, H4IIE cells and HepG2 cells exhibit relatively low levels of LCFA oxidation (56), thus making increased synthesis of phospholipid the most likely fate of diacylglycerol in the FAT/CD36 transfected cells. The effect of truncating the C terminus of FAT/CD36 on the fate of absorbed LCFAs makes it appealing to speculate that signaling events involving this domain may be responsible or, alternatively, that the precise intracellular localization of FAT/CD36 is critical for the normal channeling of newly acquired LCFAs.

In conclusion, this study demonstrates an important role for the C terminus of FAT/CD36 in determining the subcellular distribution of the molecule. Furthermore, either directly or through its influence on subcellular distribution, this domain influences the fate of newly acquired LCFAs. These results suggest that there may be cell type- or tissue-specific differences in the role of FAT/CD36 in LCFA metabolism and that these could be reflected in whether the molecule is dependent on caveolin-1 for localization to the cell surface and to lipid rafts. Further investigations into the role of the C terminus of FAT/CD36 in the subcellular localization of the molecule may help us to understand the mechanisms involved in physiological translocation of the molecule from intracellular stores to the plasma membrane in stimulated adipose tissue and muscle.

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