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Caveolin-1 is required for fatty acid translocase (FAT/CD36) localization and function at the plasma membrane of mouse embryonic fibroblasts

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Abstract

Several lines of evidence suggest that lipid rafts are involved in cellular fatty acid uptake and influence fatty acid translocase (FAT/CD36) function. However, it remains unknown whether caveolae, a specialized raft type, are required for this mechanism. Here, we show that wild-type (WT) mouse embryonic fibroblasts (MEFs) and caveolin-1 knockout (KO) MEFs, which are devoid of caveolae, have comparable overall expression of FAT/CD36 protein but altered subcellular FAT/CD36 localization and function. In WT MEFs, FAT/CD36 was isolated with both lipid raft enriched detergent-resistant membranes (DRMs) and detergent-soluble membranes (DSMs), whereas in cav-1 KO cells it was exclusively associated with DSMs. Subcellular fractionation demonstrated that FAT/CD36 in WT MEFs was localized intracellularly and at the plasma membrane level while in cav-1 KO MEFs it was absent from the plasma membrane. This mistargeting of FAT/CD36 in cav-1 KO cells resulted in reduced fatty acid uptake compared to WT controls. Adenoviral expression of caveolin-1 in KO MEFs induced caveolae formation, redirection of FAT/CD36 to the plasma membrane and rescue of fatty acid uptake. In conclusion, our data provide evidence that caveolin-1 is necessary to target FAT/CD36 to the plasma membrane. Caveolin-1 may influence fatty acid uptake by regulating surface availability of FAT/CD36.

Keywords: Lipid raft; Caveolin-1; Caveolae; Fatty acid translocase

1. Introduction

Uptake of long-chain fatty acids (LCFAs) is essential for many cellular functions and is achieved by a concert of co-existing mechanisms that probably varies in different cell types. In adipocytes, permeation of LCFAs across the plasma membrane relies on a high affinity, low capacity carrier-facilitated transport system [1]. The important role of fatty acid translocase (FAT/CD36) for LCFA uptake in adipocytes has been extensively studied. When overexpressed in cultured fibroblasts FAT/CD36 increases saturable, high-affinity LCFA uptake [2]. Moreover, FAT/CD36 knockout mice have increased serum fasting levels of nonesterified free fatty acids and reveal reduced uptake of oleate in isolated adipocytes [3]. Recently, we showed that in 3T3-L1 adipocytes, plasma membrane FAT/CD36 is located within detergent-resistant membranes (DRMs), which are enriched in lipid rafts components, whereas intracellular FAT/CD36 cofractionated with detergent-soluble membranes (DSMs) [4]. Indeed, evidence is accumulating that lipid rafts play a crucial role in regulating LCFA uptake [4–7]. Rafts are dynamic assemblies of sphingolipids and cholesterol that contain a select set of membrane proteins (reviewed in [8]). Caveolae represent a morphologically identifiable subset of lipid rafts that are particularly abundant in adipocytes [9]. Their coat protein caveolin-1 is essential for the formation of the characteristic flask-shaped invaginations of the plasma membrane through a largely unknown process [10,11]. Although the physiological roles of caveolae remains uncertain, they have been suggested to participate in a large number of important cellular functions including the regulation of cellular cholesterol homeostasis [12,13] and signal transduction (reviewed in [14]). Recently, several reports suggested that caveolin-1 might be involved in lipid homeostasis

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[15–18]. Photoaffinity labeling has identified caveolin-1 as a major plasma membrane fatty-acid binding protein in adipocytes [15]. Furthermore, caveolin-1 has been shown to move from the plasma membrane to lipid droplets in response to free fatty acids [16–18]. A role for caveolin-1 in trafficking of lipids was also supported by reports on the phenotype of caveolin-1-deficient mice [7,19]. Depletion of caveolin-1 or deletion of the caveolin-1 gene resulted in a complete loss of caveolae and significant white adipose tissue atrophy with age. In addition, these mice have a lean phenotype and show severely elevated serum levels of free fatty acids and triglycerides, especially in the postprandial state [7].

The possible role of lipid rafts in lipid flux has generated considerable interest and activity in elucidating the protein composition of these microdomains. While the overall biochemical composition of caveolae and non-caveolar lipid rafts is thought to overlap, these microdomains are not completely equivalent. In addition to the caveolins, several other proteins have been shown to preferentially localize to either caveolae or non-caveolar lipid rafts [20]. Based on its partitioning into DRM fractions, FAT/CD36 was suggested to associate with lipid raft microdomains [4,5,21], but it remains to be determined whether it is in caveolae.

In the present study, we used wild-type and caveolin-1 knockoutmouse embryonic fibroblasts (cav-1 KO MEFs) as a model to define the precise localization and regulation of FAT/CD36. We found that FAT/CD36 function relies on caveolin-1 expression. Deficiency of caveolin-1 resulted in a complete loss of caveolae, absence of FAT/CD36 plasma membrane expression and reduction of fatty acid uptake. Adenoviral expression of caveolin-1 in cav-1 KO MEFs induced re-formation of caveolae, targeting of FAT/CD36 to the plasma membrane and rescue of fatty acid uptake. It is conceivable that the lack of FAT/CD36 stabilization at the plasma membrane might contribute to the metabolic phenotype of the cav-1 null mice.

2. Materials and methods

2.1. Reagents and antibodies

[^{3}H]Oleic acid was purchased from Biotrend (Cologne, Germany). Fatty acid free BSA (fraction V), dexamethasone, phloretin, EGTA, protease inhibitors sucrose, EDTA, chymostatin, HOSu(SO_3)Na, dicyclohexylcarbodiimide, *N*,*N*-dimethylformamide (DMF) and non-radio-labeled oleic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Ultima-Gold scintillation fluid was purchased from Packard (Groningen, The Netherlands). DMSO was from Merck (Darmstadt/ Germany). Tr3-Isobutyl-1-methylxanthin was from Calbiochem (Bad Soden, Germany).

Antibodies and their sources were as follows: anti-caveolin-1 and anti-flotillin (BD Transduction laboratories), anti-Na,K-ATPase and anti-calreticulin (Novus Biological), anti-transferrin (Zymed Laboratories), anti-gp27/gp26 (kind gift of Dr. M. Dominguez, Department of Anatomy and Cell Biology, McGill University, Montreal, Canada).

Primary polyclonal antibodies against mouse FAT/CD36 were generated using the synthetic peptide SYKGKRNLSYWPSYC to which a cysteine residue had been added before coupling to keyhole limpet hemocyanin. The antiserum was produced by injection of guinea pigs (Peptide Specialty Laboratories, Heidelberg, Germany) and specifically detects FAT/CD36 protein [4]. Preincubation of the antiserum with the synthetic peptide ablated reaction of the antiserum with FAT/CD36 by western blotting and immunofluorescence, and pre-immune serum did not show any reactivity in these assays.

2.2. Animals and tissues

Cav-1 KO mice were described previously [10]. Mice homozygously null for the caveolin-1 gene were compared to wild type littermates at the age of 3 months. Mice were sacrificed by cervical dislocation and inguinal adipose tissues were rapidly excised. All animal experiments were approved by local authorities in accordance with criteria outlined by the American Physiological Society.

2.3. Cell culture

Mouse embryonic fibroblasts (MEFs) were prepared from 13.5 p.c. embryos obtained by homozygous crossings of cav-1 KO mice or WT mice [10]. MEFs were immortalized according to the 3T3 protocol by passaging them continuously until growth rates in culture resumed the rapid rates seen in early passage MEFs. MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FCS, Gibco BRL), 2 mM L-glutamine, 100 units/L penicillin, and 100 μg/L streptomycin at 37 °C in 10% CO_2.

2.4. Synthesis of and treatment with sulfosuccinimidyl-oleate (SSO)

The synthesis of sulfosuccinimidyl derivatives of oleate (SSO) was performed as described by Harmon et al. [22]. SSO treatment of differentiated MEFs was applied using a modified protocol of Abumrad and coworkers [23]. Briefly, MEF monolayers in 5 cm Ø culture dishes were washed three times with KRH buffer containing 0.2% fatty acid-free bovine serum albumin and glucose (2 mM) and then incubated with 400 μmol SSO for 30 min at 37 °C. To avoid toxicity of DMSO, the concentration of DMSO was always kept below 0.05%. At the end of the incubation, MEFs were washed three times with KRH buffer containing 0.2% fatty acid-free BSA in order to remove any unbound sulfosuccinimidyl-oleate. Afterwards, cells were resuspended for assay of oleate transport.

2.5. Assay for [^{3}H]oleic acid uptake

The [^{3}H]oleic acid uptake assays were performed as described previously [24] using confluent MEFs. Briefly, trace amounts of [^{3}H]oleic acid mixed with 173 μmol/L non-radioactive oleic acid were dissolved in a defatted BSA solution (173 μmol/L) at a ratio of 1:1. Two mL of the oleate/BSA solution were incubated with each MEF monolayer in a 5 cm Ø culture dish at 37 °C for 20 s. The uptake was stopped by removal of the solution followed by addition of 5 mL of an ice-cold stop solution containing 0.5% (w/v) albumin and 200 μM phloretin. The stop solution was discharged after 2 min, and the culture dishes were washed by dipping them six times in ice-cold incubation buffer. NaOH (2 mol/L) was added to lyse the cells, and aliquots of the lysate were used for protein and radioactivity determination. Radioactivity was determined after the addition of 10 mL of Ultima-Gold in a 1217 Rackbeta liquid scintillation counter (LKB-Wallac, Turku, Finland).

2.6. Isolation of detergent-resistant membranes

Detergent extraction with Triton X-100 was performed as described previously [25]. MEFs were rinsed with ice-cold PBS and scraped on ice into 300 μL of 1% Triton X-100, 25 mM Tris–HCl (pH 7.4), 150 mM NaCl, 3 mM EDTA (TNE) buffer containing leupeptin, pepstatin, chymostatin, and antipain (each at 25 μg/mL). Cells were homogenized 15 times through a 22 gauge needle followed by 10 strokes with a tight fitting Dounce homogenizer. The lysate was centrifuged for 5 min at 3000 rpm to obtain a postnuclear supernatant. The supernatant was adjusted to 40% sucrose and overlaid with a discontinuous sucrose gradient (6 mL of 30% sucrose in TNE or 2 mL of TNE without sucrose). The gradients were centrifuged at 200,000 × *g* in a Beckman SW41 rotor for 16–22 h at 4 °C. Fractions (1 mL each) were obtained and used for liquid scintillation counting and Western blotting.

2.7. Fractionation of total membranes by discontinuous OptiPrep step gradient

Fractionation of the membranes was performed using the OptiPrep gradient by AXIS-Shield (AXIS-Shield, Oslo, Norway) according to the instructions of the manufacturer. Briefly, cells were washed once with PBS to remove the culture medium and then resuspended in homogenization buffer (sucrose 25 mM, EDTA 0.5 mM, Tris 10 mM). Afterwards, cells were scraped in 3 mL homogenization buffer and centrifuged at 1000×*g* for 5 min. The pellet was resolved in 2 mL homogenization buffer and cells were lysed by 10 passages through a fine syringe needle followed by treatment with a tight-fitting Dounce homogenizer. The homogenate was centrifuged at 1000×*g* for 10 min to obtain a postnuclear supernatant. The supernatant was centrifuged at 100,000×*g* for 40 min and resuspended in 1 mL homogenization buffer containing 25% (w/v) iodixanol. The cell suspension was loaded on a nine-step OptiPrep gradient consisting of 25, 22, 19, 16, 13, 10, 7, 4, 1% (w/v) iodixanol solutions. Centrifugation was done in Beckman SW 41Ti rotor at 200,000×*g* for 3 h at 4 °C. Eighteen fractions were collected from the bottom of each centrifuge tube. A quarter of each fraction was analyzed with SDS-PAGE and Western blotting.

2.8. Adenoviral infection

The adenovirus encoding caveolin-1 tagged with fused EGFP in its C-terminus was constructed according to He et al. [26]. cDNA of caveolin-1 GFP [27] was subcloned into the shuttle vector pAd Track-CMV using *Bgl*II-Not I sites. The adenoviral vectors were propagated in HEK 293 cells, purified on Optiprep gradients and stored at −80 °C.

Incubation of MEFs with the adenovirus in serum-free medium for 4 h at 37 °C followed by growth in standard medium for 2 days resulted in a 70–80% rate of infection (as judged by GFP fluorescence). Afterwards, cells were processed for membrane fractionation. YFP-GL-GPI adenovirus was used for mock infection [27].

2.9. RNA preparation and Real-time quantitative RT-PCR

RNA isolation, cDNA synthesis, and quantitative RT-PCR were done as described [28]. The levels of GAPDH mRNA was used as an internal standard to determine relative mRNA levels of the target genes in WT and cav-1 KO adipose tissues. Reverse and forward primers used for CD36 are $^{5'}$GATGTGGAACCCATAACTGGATTCAC$^{3'}$ and $^{5'}$GGTCCCAGTCTCATTTAGCCAC AGTA$^{3'}$, and for GAPDH $^{5'}$CAAGGTCATCCATGACAACTTTG$^{3'}$ and $^{5'}$GGCCATCCACAGTCTTCTGG$^{3'}$.

2.10. Western blot analysis

Aliquots of membrane fractions or total lysate from inguinal adipose tissues were separated with SDS-PAGE and transferred to nitrocellulose membranes. Antibody binding was visualized using the ECL reagents (Amersham). Immunoreactive bands on autoradiography films were scanned (Epson GT 9600; Epson, Tokyo, Japan) and quantified using Raytest image software (Raytest, Straubenhardt, Germany).

2.11. Immunocytochemistry

MEFs were washed 3 times in PBS and fixed in 2% paraformaldehyde. The antiserum against FAT/CD36 described above was applied for 4 h at a dilution of 1:50 at 4 °C followed by washing 3 times with 0.05 mmol/L Tris–HCl, pH 7.4. The monoclonal antibody against caveolin-1 was from BD Transduction Laboratories (Heidelberg, Germany) and used at a dilution of 1:100 overnight. The secondary Cy2-conjugated antibodies against mouse and guinea pig (Dianova, Hamburg, Germany) were applied for 2 h at 4 °C diluted 1:200 in 0.05 mmol/L Tris–HCl, pH 7.4.

2.12. Electron microscopy

MEFs were grown on sapphire coverslips and rapidly frozen in 20% BSA in medium using an EMPACT2 (Leica Microsystems) containing a rapid transfer system (RTS) to facilitate loading and freezing. The frozen samples were freeze substituted in aceton containing 1% osmium tetra-oxide and 0.1 uranyl acetate using an AFS (Leica Microsystems) and afterwards embedded in Epon. Ultrathin sections were counterstained according to standard procedures.

2.13. Statistical analysis

Results are given as means ± S.D. of at least five observations. Student's *t*-test was used to test for significant differences between means.

3. Results

3.1. Effect of caveolin-1 deficiency on FAT/CD36 expression

In order to determine if caveolin-1 influences FAT/CD36 mRNA levels or protein stabilization, we first studied FAT/CD36 expression in inguinal adipose tissues from WT and cav-1 KO mice. Fig. 1A shows that there was no difference between FAT/CD36 protein expression and mRNA levels in WT and cav-1 KO adipose tissues. Next, we analyzed FAT/CD36 in MEFs isolated from WT or cav-1 KO embryos and found that the FAT/CD36 protein and mRNA content in whole cell lysates was comparable (Fig. 1B). From these data, we conclude that caveolin-1 neither affects FAT/CD36 mRNA levels nor FAT/CD36 protein stability.

In addition, the quantitative mRNA expression of FAT/CD36 in inguinal adipose tissue and MEFs ranged in the same order of

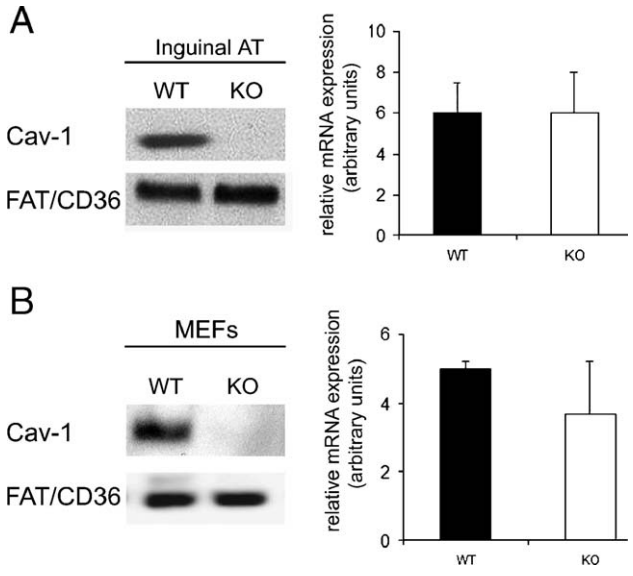

Fig. 1. FAT/CD36 expression in inguinal adipose tissues and MEFs derived from WT and Cav-1 KO mice. (A) Expression of caveolin-1 and FAT/CD36 in inguinal adipose tissues from WT and cav-1 KO mice. For western blotting, 20 μg of total lysate was loaded per lane. FAT/CD36 mRNA expression was assessed by real time PCR as described in Materials and methods. The expression was normalized against expression of GAPDH. As expected, caveolin-1 was not detected in adipose tissues from cav-1 KO mice. No significant changes were observed between FAT/CD36 expression at either the protein or mRNA levels. (B) Expression of caveolin-1 and FAT/CD36 in MEFs derived from embryos from WT and cav-1 KO mice. A total of 50 μg of protein was applied per lane. As expected, caveolin-1 expression was ablated in the cav-1 KO MEFs. Expression of FAT/CD36 at either the protein or mRNA levels was comparable in WT and cav-1 KO MEFs.

magnitude, suggesting that MEFs are a suitable cell culture system to study FAT/CD36 localization and function.

3.2. FAT/CD36 is absent from lipid raft-enriched membrane fractions in cav-1 KO MEFs

FAT/CD36 has been shown to associate with detergent-resistant membranes (DRMs), which are enriched in lipid rafts components. To investigate if caveolin-1 influences FAT/CD36 partitioning in DRM fractions, total lysates of WT and cav-1 KO MEFs were extracted by Triton X-100 and floated on sucrose gradients. Afterwards, fractions collected from the gradient were analyzed by immunoblotting. Flotillin and caveolin-1 were used as marker proteins for DRM fractions and the transferrin receptor served as a marker for detergent-soluble membrane (DSM) fractions (Fig. 2). In WT MEFs, FAT/CD36 was expressed in both DRM and DSM fractions. In contrast, analysis of fractions from cav-1 KO MEFs revealed that FAT/CD36 was present in DSMs but completely absent from DRMs. A similar amount of total protein was recovered from DRM and DSM fractions in WT (1.1± 0.09 mg and 4.6±1.2 mg, respectively) and cav-1 KO MEFs (1.1±0.8 mg and 3.9±0.6 mg, respectively) showing that the disappearance of FAT/CD36 from the DRM fractions in cav-1 KO MEFs was not due to a lower overall recovery of proteins in these cells.

3.3. Caveolin-1 is required for stabilization of FAT/CD36 at the plasma membrane

These findings indicated that caveolin-1 might be required as an anchor for FAT/CD36 in DRMs. As plasma membrane FAT/CD36 in 3T3-L1 adipocytes had previously been shown to be located exclusively in DRMs [4], we hypothesized that in the absence of caveolin-1 FAT/CD36 might be retained intracellularly and not be expressed on the plasma membrane at all. To further test this hypothesis, we performed immunofluorescence microscopy to examine the localization of FAT/CD36 in WT and cav-1 KO MEFs. Fig. 3 shows that in WT MEFs, FAT/CD36 is mainly located at the plasma membrane along the cell–cell contact sites whereas in cav-1 KO cells it is exclusively found in the cytoplasm. To corroborate this finding, we fractionated WT and cav-1 KO MEFs homogenates on a discontinuous iodixanol OptiPrep step gradient in order to separate fractions containing plasma membrane (nos. 3–7), Golgi-network (nos. 8–14) and endoplasmic reticulum (nos. 14–18) (Fig. 4A). Na-K-ATPase, gp26/27 and calreticulin, respectively, served as markers of these fractions. In WT MEFs, FAT/CD36 was present in the plasma membrane and Golgi fractions (Fig. 4B). In contrast, in cav-1 KO MEFs FAT/CD36 was absent from plasma membrane fractions but was found in an intracellular pool within the Golgi network fractions.

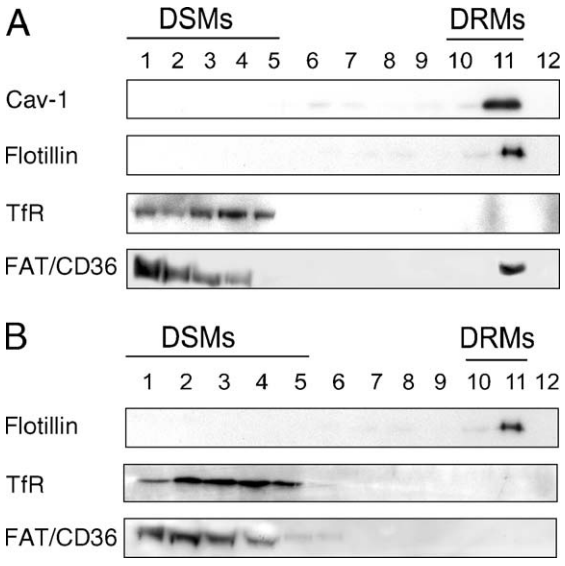

Fig. 2. Expression of FAT/CD36 in lipid raft-enriched membrane fractions. WT MEFs and cav1 KO MEFs were lysed in cold 1% Triton X-100, and the total lysate was resolved on a sucrose density gradient by ultracentrifugation. Fractions 1–12 (fraction 12 representing the top of the gradient) were collected, separated by SDS-PAGE, and blotted with antibodies to caveolin-1 and flotillin (marker proteins of DRMs), the transferrin receptor (marker protein of DSMs) and FAT/CD36. While in WT MEFs, (A) FAT/CD36 was present in both DRMs and DSMs, in cav-1 KO MEFs (B) it was excluded from DRMs.

To investigate if expression of recombinant caveolin-1 is sufficient for FAT/CD36 targeting to the plasma membrane, we used a cav1-GFP adenovirus to restore caveolin-1 expression in cav-1 KO MEFs. 48 h after infection, ~70% of cells expressed GFP (data not shown) and the amount of recombinant caveolin-1 expressed in cav-1 KO MEFs (rCav-1) was comparable to the endogenous level in WT MEFs (Fig. 5A). Adenoviral expression of caveolin-1-GFP in cav-1 KO cells was sufficient to induce caveolae formation as shown by electron microscopy (Fig. 5B). Membrane fractionation revealed that expression of recombinant caveolin-1 in cav-1 KO MEFs shifted the cellular distribution of FAT/CD36 from the Golgi compartment to the plasma membrane (Fig. 5C). As a negative control for this experiment, cav-1 KO cells were infected with a control adenovirus encoding YFP-GL-GPI (YFP-tagged glycosylated form of glycosyl phosphatidyl inositol anchor), known to associate with DRMs. Importantly, this mock infection did not redirect FAT/CD36 to the plasma membrane (Fig. 5C).

3.4. Fatty acid uptake parallels FAT/CD36 plasma membrane expression

Since FAT/CD36 is known to play a major role in LCFA incorporation, we next analyzed the effect of FAT/CD36 mislocalization in cav-1 KO MEFs on fatty acid uptake. We found that uptake of [^{3}H]-oleic acid bound to albumin (173 μmol/L) over 20 s was reduced by 61.6% in cav-1 KO compared to WT MEFs (Fig. 6). Pretreatment with SSO, a selective inhibitor of FAT/CD36 function, inhibited [^{3}H]-oleate uptake in WT MEFs by 41.1% but did not affect oleate uptake in cav-1 KO MEFs. Re-expression of caveolin-1 in cav-1 KO cells rescued fatty acid uptake nearly to levels of WT MEFs. In rCav-1 cells, pretreatment with SSO inhibited oleate uptake by almost 40%, although this effect did not reach statistical significance (P=0.07). These findings show that the contribution of FAT/CD36 to oleate uptake is abrogated in the absence of caveolin-1.

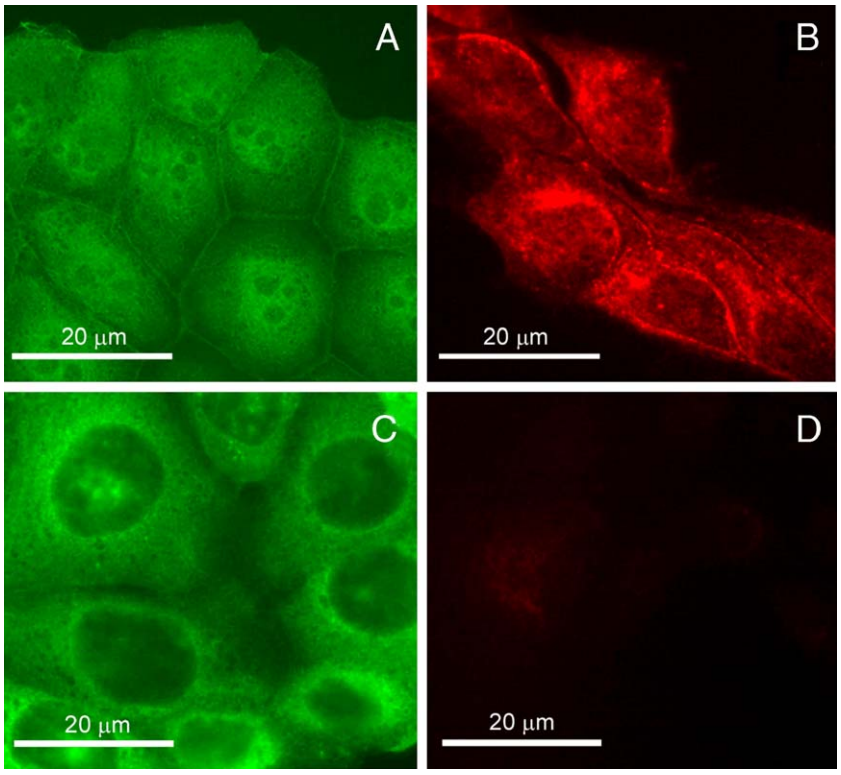

Fig. 3. Immunofluorescence staining of FAT/CD36 and caveolin-1 in WT and cav-1 KO MEFs. In wild type MEFs, FAT/CD36 (A) and caveolin-1 (B) antibodies stain the plasma membranes along the cell-cell borders. In contrast, CD36 is found exclusively in the cytosolic compartment of cav-1 KO MEFs and does not stain the plasma membrane (C). Panel D shows the absence of caveolin-1 staining in the cav-1 KO MEFs.

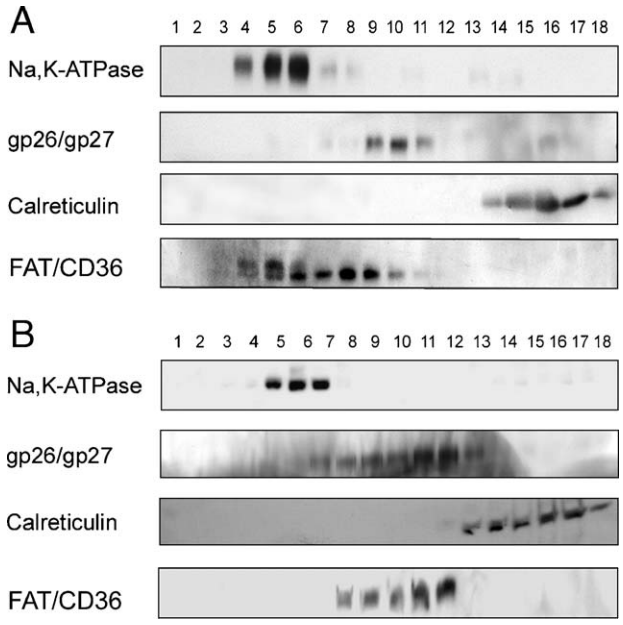

Fig. 4. FAT/CD36 is absent from plasma membranes in cav-1 KO MEFs. Fractionation was performed using a discontinuous OptiPrep step gradient, and equal amounts of protein were separated by SDS-PAGE, blotted, and probed with antibodies against the indicated protein. The detection of marker proteins for plasma membrane (anti Na-K ATPase), golgi network (gp 26/27) and ER (calreticulin) and FAT/CD36 is shown for WT MEFs (A) and cav-1 KO MEFs (B). While FAT/CD36 is present in plasma membrane fractions of WT MEFs, it is completely absent from plasma membrane fractions of cav-1 KO MEFs.

4. Discussion

Translocation of LCFAs across the plasma membrane is achieved by a concert of co-existing mechanisms. A number of recent studies in cell lines and caveolin-1 knockout mice have suggested that lipid rafts/caveolae and their marker protein, caveolin-1, may play a crucial role in this process [4,5,7,15,29,30]. For the first time, the present study provides functional and molecular genetic evidence that caveolin-1 targets FAT/CD36 to plasma membrane caveolae where it is necessary for proper stabilization of FAT/CD36 at the cell surface. In the absence of caveolin-1, FAT/CD36 is retained in an intracellular pool that cofractionates with elements of the Golgi network. Expression of caveolin-1 in cav-1 KO cells is sufficient to redirect FAT/CD36 to the cell surface. The functional significance of this finding is underlined by the fact that the reduced [^{3}H]oleate uptake of cav-1 KO MEFs was reversed by caveolin-1 re-expression.

These data may shed a new light on the pathogenesis of adipose tissue atrophy and elevated serum levels of free fatty acids observed in cav-1 KO mice (19). Cohen et al. [31] proposed that these phenotypes might be due to reduced plasma membrane expression of the insulin receptor resulting in enhanced lipolysis and diminished lipogenesis. However, there is conflicting data concerning the effects of caveolin on insulin receptor expression and function [32,33]. Our results suggest that FAT/CD36 deficiency at the cell surface might, at least in part, contribute to the metabolic changes observed in cav-1 KO mice. Interestingly, FAT/CD36 knockout mice resemble cav-1 null KO mice in that they

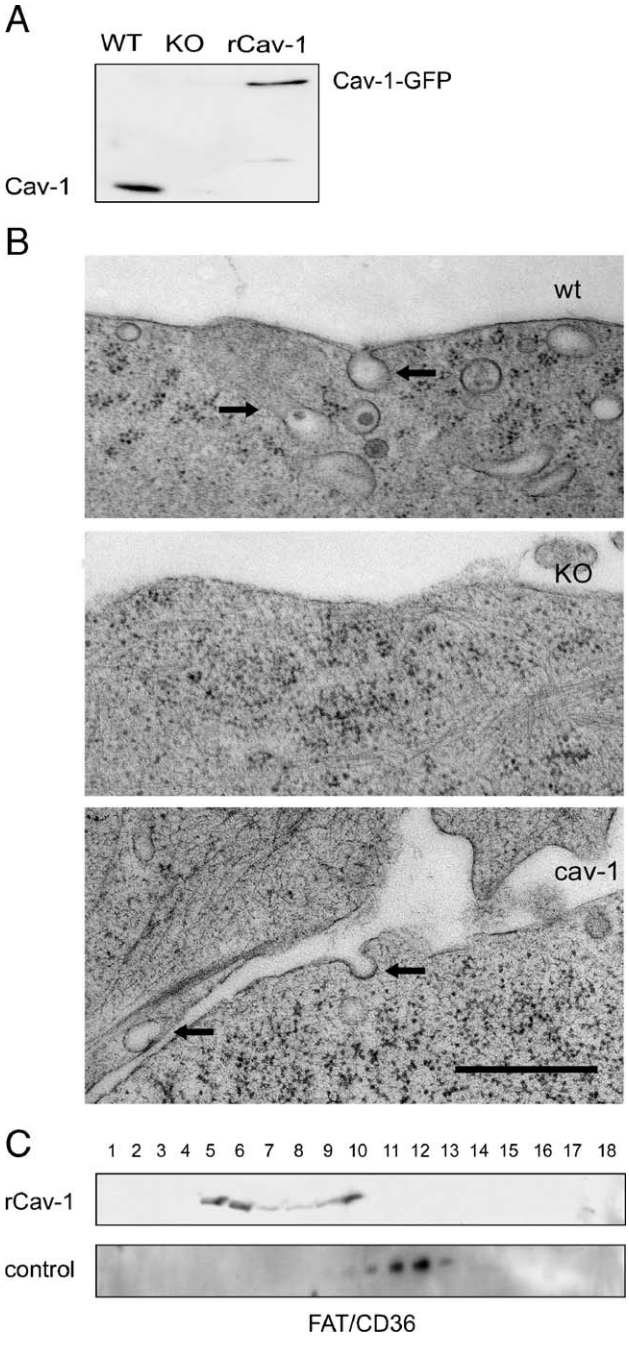

Fig. 5. Recombinant expression of caveolin-1 rescues plasma membrane caveolae and FAT/CD36 expression. (A) Protein expression of endogenous caveolin-1 in WT MEFs and KO MEFs and expression of recombinant caveolin-1-GFP in cav-1 KO MEFs, as assessed by Western Blot with caveolin-1 antibody. While endogenous caveolin-1 can be detected as a 22 kDa protein in WT MEFs, after transient expression of caveolin-1 in KO MEFs (rCav-1) caveolin-1-GFP can be identified as a 45 kDa protein. A total of 50 μg of protein was applied per lane. (B) Electron micrographs of the plasma membrane of MEFs. Cav-1 KO MEFs are devoid of caveolae structures. Re-expression of caveolin-1 allows re-formation of caveolae invaginations in the plasma membrane (arrows), as observed in WT MEFs. Scale bar, 500 nm. (C) Membrane fractionation of cav-1 KO MEFs 48 h after adenovirus mediated expression of recombinant caveolin-1 (rCav-1) shows a shift of FAT/CD36 localization to the plasma membrane. In contrast, mock infection with an adenovirus encoding YFP-GL-GPI had no effect on FAT/CD36 localization in cav-1 KO MEFs.

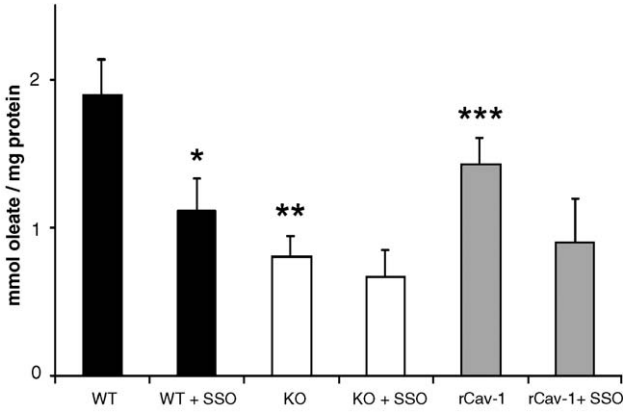

Fig. 6. Effects of SSO and caveolin-1 deficiency on [^{3}H]oleate uptake in MEFs. Pretreatment with SSO (400 μM for 30 min at 37 °C), a selective inhibitor of FAT/CD36 function, inhibited oleate uptake over 20 s in WT MEFs by 41.1%. In cav-1 KO MEFs, oleate uptake was reduced by 61.6% compared to WT MEFs. Pretreatment with SSO had no relevant effect on oleate uptake in cav-1 KO MEFs. Re-expression of caveolin-1 in cav-1 KO cells (rCav-1) rescued fatty acid uptake close to the levels of WT MEFs. In rCav-1 cells, pretreatment with SSO inhibited oleate uptake by almost 40%, although this was not statistically significant ($P=0.07$). Values are means ± SD of five independent experiments. *$P<0.05$ for untreated vs. SSO-treated WT MEFs; **$P<0.05$ for Cav1 KO vs. WT MEFs; ***$P<0.05$ for rCav-1 vs. cav-1 KO MEFs.

display increased levels of free fatty acids and reduced uptake of oleate in isolated adipocytes [3]. However, these knockout mice differ substantially on some other parameters. Most importantly, insulin resistance or adipose tissue atrophy have not been observed in FAT/CD36 deficient mice. Therefore, malfunction of FAT/CD36 does not fully explain the phenotype of cav-1 KO mice.

An important property of caveolae is that they include and exclude proteins to a various extent thus achieving a spatial segregation of membrane processes. We and others have previously reported that FAT/CD36 is enriched in lipid rafts of adipocytes [4,5,34]. However, contradictory results as to whether FAT/CD36 is present in caveolar rafts or not have been reported. Based on confocal fluorescence microscopy studies, Zeng et al. [21] suggested that in CHO cells FAT/CD36 is localized in lipid rafts but not in caveolae. In contrast, Souto and coworkers [34] immunoisolated caveolae from adipocytes and found FAT/CD36 copurified with caveolin-1 and subsequently confirmed these findings by electron microscopical analysis. Frank and coworkers [35] reported that in Cos7 cells FAT/CD36 was targeted to the plasma membrane only when cotransfected with caveolin-1. In the absence of caveolin-1, FAT/CD36 was retained intracellularly in a perinuclear Golgi-like compartment, as assessed by immunocytochemistry [35]. Finally, the data from Uittenbogaard et al. [36] show that in human microvascular endothelial cells, CD36 and caveolin-1 co-precipitate together. Our data show that the expression of FAT/CD36 on the plasma membrane and in part the uptake of oleate depend on the presence of caveolin-1. In contrast to the studies by Uittenbogaard et al. [36] in endothelial cells, we found that caveolin-1 and FAT/CD36 did not precipitate together in MEFs (data not shown), suggesting that FAT/CD36 does not directly interact with the scaffolding domain of caveolin-1, which is a known binding region for different proteins within caveolae [37,38]. The mechanism by which caveolin-1 stabilizes FAT/CD36 within plasma membrane

caveolae remains unclear. FAT/CD36 is a transmembrane protein with a very short cytoplasmic tail comprising 5–8 amino residues. Souto and colleagues [34] suggested that oligomerized caveolin covers the cytoplasmic surface of caveolae and therefore only membrane proteins with small cytoplasmic domains would have free access to caveolae, whereas proteins with large cytoplasmic domains would be excluded by steric limitations.

Little is known about the regulation of FAT/CD36 function, but in muscle cells, several lines of evidence point toward a translocation mechanism for increasing LCFA uptake by FAT/CD36 [39–41]. Muscle cells express a specific type of caveolin, caveolin-3, which might play a similar role for FAT/CD36 translocation as caveolin-1 in MEFs. Future investigations will address the question if caveolin-3 might be responsible for translocation of FAT/CD36 to muscle cell plasma membranes. We propose a model in which FAT/CD36 recycles from intracellular non-DRM pools to caveolae at the plasma membrane. Caveolin-1 may control FAT/CD36 surface availability and thereby indirectly regulate fatty acid uptake. After FAT/CD36 mediated binding of LCFAs at the outer leaflet of the plasma membrane, LCFAs translocate through the membrane bilayer. Based on previous reports [7,16,17], it is conceivable that LCFAs translocating to the inner leaflet of adipocyte plasma membranes may be bound by caveolin-1 that might serve as an intracellular shuttle trafficking LCFAs to lipid droplets. This issue needs to be addressed in further studies.

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