

Research Paper

Overexpression of CD36 and Acyl-CoA Synthetases FATP2, FATP4 and ACSL1 Increases Fatty Acid Uptake in Human Hepatoma Cells

Julia Krammer, Margarete Digel, Friedrich Eehalt, Wolfgang Stremmel, Joachim Füllekrug and Robert Eehalt ✉

Department of Gastroenterology, Hepatology and Infectious Diseases, University Hospital Heidelberg, Im Neuenheimer Feld 410, 69120 Heidelberg, Germany

✉ Corresponding author: Prof. Dr. med. Robert Eehalt, Department of Gastroenterology, University Hospital Heidelberg, Im Neuenheimer Feld 410, 69120 Heidelberg; Robert.Eehalt@med.uni-heidelberg.de; Telephone: +49(0)62215638715, Fax: +49(0)6221565255.

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Abstract

Background: Understanding the mechanisms of long chain fatty acid (LCFA) uptake in hepatic cells is of high medical importance to treat and to prevent fatty liver disease (FLD). ACSs (Acyl-CoA synthetases) are a family of enzymes that catalyze the esterification of fatty acids (FA) with CoA. Recent studies suggest that ACS enzymes drive the uptake of LCFA indirectly by their enzymatic activity and could promote special metabolic pathways dependent on their localization.

The only protein located at the plasma membrane which has consistently been shown to enhance FA uptake is CD36.

Aims: The current study investigated whether ACSs and CD36 could regulate hepatic LCFA uptake.

Methods and Results: FATP2 and FATP4 were both localized to the ER of HuH7 and HepG2 cells as shown by double immunofluorescence in comparison to marker proteins. ACSL1 was located at mitochondria in both cell lines. Overexpression of FATP2, FATP4 and ACSL1 highly increased ACS activity as well as the uptake of [³H]-oleic acid and fluorescent Bodipy-C12 (B12) fatty acid. Quantitative FACS analysis showed a correlation between ACS expression levels and B12 uptake. FATP2 had the highest effect on B12 uptake of all proteins tested. CD36 was mainly localized at the plasma membrane. Whereas [³H]-oleic acid uptake was increased after overexpression, CD36 had no effect on B12 uptake.

Conclusion: Uptake of LCFA into hepatoma cells can be regulated by the expression levels of intracellular enzymes. We propose that ACS enzymes drive FA uptake indirectly by esterification. Therefore these molecules are potential targets for treatment of nonalcoholic fatty liver disease (NAFLD) or steatohepatitis (NASH).

Key words: fatty acid transport, acyl-CoA synthetases, nonalcoholic fatty liver disease, nonalcoholic steatohepatitis, CD36

INTRODUCTION

The molecular mechanism of long chain fatty acid (LCFA) uptake is still a matter of debate (for reviews see [1-8]). Understanding this process is important to elucidate potential targets for the treatment of lipid disorders like nonalcoholic fatty liver disease

(NAFLD), which is caused by an accumulation of triglycerides in the liver. A common feature for the hepatic triglyceride accumulation is an elevated plasma level of free fatty acids (FFA) found especially in correlation with obesity and insulin resistance [9, 10].

Studies suggest that the rate of hepatic FFA uptake is directly proportional to plasma FFA concentrations [11]. Obesity results in a marked enlargement of the intraabdominal visceral fat. The development of insulin resistance leads to an elevated rate of lipolysis of these depots. Released LCFAs are transported to the liver where they are translocated across the plasma membrane, reassembled to triglycerides and stored in lipid droplets [12]. To interfere with the uptake mechanism is therefore a promising target for further therapeutic intervention for one of the major burdens of our society. The prevalence of hepatic steatosis in the general population is already estimated between 14 % and 24 % with an increasing tendency in all segments of the population [13, 14].

The first step of LCFA uptake is its translocation across the plasma membrane. There is a high degree of controversy regarding the mechanism of LCFA translocation. However, the concept of a facilitated uptake by special membrane components like proteins and lipid rafts had become popular in recent years [1, 3, 8]. There have been different candidate proteins published that are possibly involved in LCFA uptake of which only CD36 has consistently been shown to be expressed at the plasma membrane and to enhance LCFA uptake upon overexpression [4]. CD36 is a scavenger protein that besides FAs can bind a variety of molecules including acetylated LDL, collagen and phospholipids [4, 15-18]. It is located in lipid rafts and non-raft domains of the cellular plasma membrane and most likely facilitates LCFA transport by accumulating LCFA on the outer surface [3, 19, 20]. As CD36 has been shown to be expressed in liver tissue [21, 22] as well and is upregulated in nonalcoholic fatty liver disease (NAFLD) [23] and nonalcoholic steatohepatitis (NASH) [22], we examined its capability for LCFA uptake in our systems.

The first step for intracellular use of LCFA is its esterification with coenzyme A (CoA). This reaction is catalyzed by acyl-CoA synthetases (ACs). The produced acyl-CoAs are substrates for β -oxidation, the synthesis of triacylglycerol, phospholipids, cholesterol esters as well as ceramides and are also a primary source of signaling molecules [2, 24]. In humans two related protein families for long chain fatty acid activation exist: The fatty acid transport proteins (FATPs) and the long chain acyl-CoA synthetases (ACSLs) [25, 26]. They differ in tissue expression and in the cellular localization. Thereby they likely play a role in channeling LCFA to different intracellular metabolic pathways [2, 27]. As the overexpression of most of these proteins leads to an enhanced LCFA uptake [19, 28-30], these enzymes are potential pharmacological targets. In this study we investigated ACSL1, FATP2

and FATP4, all proteins that have been shown to be expressed in human liver [22, 23, 31-35].

We set the goal to better understand the role of FATP2, FATP4, ACSL1 and CD36 in LCFA uptake and lipid metabolism of hepatocytes to point out potential targets for treatment of hepatic steatosis.

MATERIALS AND METHODS

Reagents and Antibodies

Antibodies used were mouse anti-placenta alkaline phosphatase (PLAP) (Dako Cytomation), mouse anti-FLAG (Sigma), mouse anti-HA (Santa Cruz), Allophycocyanin (APC), Cy2 and Cy3 donkey anti-mouse (Jackson Immuno Research).

Constructs

GFP-FATP4 and FATP4-FLAG have been described previously [29]. CD36-FLAG was kindly provided by Douglas M. Lublin, Washington University School of Medicine, St. Louis, MO. FLAG epitope-tagged ACSL1 [36] was a gift from Rosalind Coleman, PLAP cDNA [37] and HA epitope-tagged FATP2 were from our laboratory.

Plasmid constructs used for localization studies were pOCT-GFP [38], a gift from Heidi McBride and mito-RFP (containing the N terminus of rat aldehyde dehydrogenase fused to mRFP) as markers for mitochondria. ADRP-mRFP1, a marker for lipid droplets, mRFP-sec61 β , a marker for ER and peroxisomal-RFP, a marker for peroxisomes were gifts of Christoph Thiele and Lars Kürschner, MPI Molecular Cell Biology, Dresden, Germany. From our laboratory ssGFPglycTMDpcx as well as PLAP, both constructs for plasmamembrane marking, were used.

Oleate Uptake

Cumulative uptake of oleate was based on Stremmel and Berk 1986 [39]. Adherent cells were incubated for 5 min at 37 °C with [3H]oleate uptake solution (170 μ M [3H] oleate, 170 μ M BSA in PBS; specific activity 4.1 Ci/mol). After stopping and washing with ice-cold 0.5 % BSA in PBS, cells were lysed with 1 M NaOH and aliquots analyzed by scintillation counting (Biorad) for protein concentration and radioactivity as before [40].

Cells and transient transfection

HuH7 (JCRB 0403) and HepG2 (ATCC HB-8065) cells were maintained under standard tissue culture conditions with the appropriate culture media (HuH7: D-MEM Invitrogen; 4,5 g glucose/L, 10% FBS, 2 mM L-Glutamine and HepG2: D-MEM Invitrogen; 4,5 g glucose/L 10% FBS 2mM L-Glutamine, 1% So-

dium pyruvate Sigma, 1% non-essential aminoacid solution Sigma). HepG2 cells were grown on a Collagen R surface coating (Serva). Cells grown to near confluency (10 cm²) were transfected with 2 µg plasmid-DNA and 10 µl FuGENE HD (Roche). Analysis was performed 24-48 hours after transfection.

Immunofluorescence and confocal analysis

For immunofluorescence microscopy, cells were fixed for 10 min with 4% PFA at RT followed by a permeabilisation with 0.1% saponin (Sigma) for 10 min. Fixed cells were incubated for 1 hour at room temperature with the appropriate dilution of antibodies in phosphate-buffered saline (PBS) containing 0.2% gelatine and 0.1% saponin.

After three washes with PBS/ 0.2% gelatine cells were incubated with the respective fluorescently labelled secondary antibodies in PBS containing 0.2% gelatine and 0.1% saponin for 1 hour at room temperature. The antibody against PLAP and the anti-HA antibody were diluted 1:100, the anti-FLAG antibody 1:1000.

Cy3-labelled secondary antibodies were diluted 1:1000, and the Cy2-labelled ones 1:200. The cells were fixed as described above. Coverslips were mounted in Mowiol and fluorescent images were acquired on a Leica TCS SP2 confocal microscope as described in [41] and arranged with Adobe Photoshop. All confocal images are representative single sections.

B12-FA uptake and FACS analysis

Flow cytometric analyses were essentially performed as described before [29]. Transfected HuH7 and HepG2 cells were incubated with 2 µM B12-FA [Bodipy 3823 (4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoic-acid) Invitrogen] bovine serum albumin (BSA) solution for 2 min. After the termination of FA uptake by adding ice-cold 0.1% BSA in PBS, the cells were trypsinized, fixed in suspension with 4% paraformaldehyde and stained with the appropriate antibodies. For FATP2-HA, CD36-FLAG and ACSL1-FLAG staining we incubated the cells with APC (Allophycocyanin) as second antibody for 30 min. FATP4 was used as an RFP-construct. Appropriate controls (for autofluorescence, single signals) were used for calibration of the FACS analysis. Cells were analyzed with a FACS Scan and LSRII FACS (BD Biosciences). Data were exported from the CellQuestPro software (BD Biosciences, San Jose, CA) and FACS Diva Software (BD Biosciences, San Jose, CA) and analyzed by spreadsheet calculations [42]. Moreover, $S_{(Bodipy)}$ [AU] was calculated using the absolute number of cells overexpressing one of the candidate proteins (in % of all cells

gated) and the overall mean of Bodipy fatty acid fluorescence measured.

Acyl-CoA synthetase activity

The protocol is based on Hall et al. [43]. Cells were lysed for 30 min on ice with 1% TX-100, 130 mM KCl, 25 mM Tris-HCl, pH 7.4. Protein lysates were incubated for 10 min at 30°C in 100 mM Tris, pH 7.4, 5 mM MgCl₂, 200 µM dithiothreitol (DTT), 10 mM ATP, 0.2% TX-100, 20 µM [³H]oleate (specific activity 10 Ci/mol), 200 µM CoA. The reaction was stopped with Dole's solution (isopropanol: n-heptane: H₂SO₄ 40:10:1), unreacted oleate was extracted four times with n-heptane and the remaining oleoyl-CoA in the aqueous phase determined by scintillation counting.

Real time PCR

First cDNA was prepared using Transcriptor First Strand cDNA Synthesis Kit (Roche) and mRNA prepared using High Pure RNA Isolation Kit (Roche) according to the manufacturer's instructions. The final RNA concentrations were determined spectrophotometrically with BioPhotometer (Eppendorf).

Using reverse transcription quantitative PCR (qPCR) we studied FATP2, FATP4, ACSL1 and CD36 and obtained these to our housekeeping gene β-Actin.

For each gene investigated specific primers were designed for optimal performance with its specific annealing temperature by software (<http://frodo.wi.mit.edu/primer3/>) [44]. Primer pairs (self-designed) are listed below:

ratACSL1

Forward

5'-TGGGCACAGAAGAGAGGATT-3'MT(°C): 63,8

Reverse

5'- ATGGCTTCAAACCAGCATTTC-3'MT(°C): 63,9

hACSL1

Forward

5'-CTTCTGGTACGCCACGAGAC-3'MT(°C): 64,8

Reverse

5'-GTCGCTGTCAAGTAGTGCG-3'MT(°C): 62,3

hCD36

Forward

5'-TTGATGTGCAAATCCACAGG-3'MT(°C): 65,7

Reverse

5'-TGIGTTGTCTCAGCGTCCT-3'MT(°C): 66

mFATP2

Forward

5'-TCGTGGGACTGGTAGATTTTG-3'MT(°C): 64

Reverse

5'-CGCGATGTGTTGAAAGAGTTT-3'MT(°C): 63,8

hFATP2

Forward

5'-TGGTGTGCCAGAACTACAAG-3'MT(°C): 65,1

Reverse

5'-GAAAGAGTCAATCCCATCTGTGT-3'MT(°C):
63,1

mFATP4

Forward

5'-GTGAGATGGCCTCAGCTATC-3'MT(°C): 61,4

Reverse

5'-GAAGAGGGTCCAGATGCTCT-3'MT(°C): 62,3

hFATP4

Forward

5'-CGGTTCGGGACGATTGTAT-3'MT(°C): 63,6

Reverse

5'-AACCTGGTGCTGGTTTTCTIG-3'MT(°C): 64

human β -actin

Forward

5'-AGGATGCAGAAGGAGATCACT-3'MT(°C): 61,8

Reverse

5'-GGGTGTAACGCAACTAAGTCATAG-3'MT(°C):
62,5

Levels of the following thermal profile were used: Enzyme activation at 95° for 10min followed by 35 amplification cycles of 15 sec at 95° (denaturation step), 30 sec at 60° (annealing step) and 30 sec at 72° for Elongation. Paying attention the melting curves show just one peak for every sample, data acquisition occurred at the elongation step of each cycle, and the threshold cycle (Ct) for each sample was calculated by determining the point at which fluorescence exceeded the threshold limit.

For use as internal standards, control plasmids including each gene were diluted to 10^{310^7} copies. RT qPCRs were carried out in 96 well reaction plates using Power SYBR® Green PCR Master Mix from Applied Biosystems. Results were analyzed using the software by 7500 Fast Real-Time PCR System from Applied Biosystems and finally given as the relative expression to the housekeeping gene for human β -actin. Results were collected from at least two independent experiments using each time three different RNA samples and were presented as a ratio to actin expression.

Statistical analysis

All values are reported as mean and standard error of the mean (SEM). The ANOVA test was used to test for statistical significance. Probability values of $p < 0.05$ were set as threshold for statistical significance.

RESULTS

Subcellular localisation of FATP2, FATP4, ACSL1 and CD36

To study the role of FATP2, FATP4, ACSL1 and CD36 in LCFA transport of hepatoma cells we first

investigated the localization of these proteins. So far the localization of FATP2 was described in peroxisomes as well as at the plasma membrane of rat, mouse and monkey cells [27, 34, 45] and additionally in the endoplasmatic reticulum (ER) of COS cells [46]. FATP4 was originally reported to be located at the plasma membrane and act there like a transporter for LCFA [28]. However, recent studies could show a localization in the ER of mouse small intestine, HeLa, Caco-2, Ptk2, MDCK, 3T3-L1 and COS cells [29, 47]. For localization studies HA-tagged FATP2 as well as GFP-tagged FATP4 was transiently transfected in HuH7 and HepG2 cells. In these cells they showed a similar reticular cellular pattern typifying the ER membranes (figure 1 and 2). ER localization was assured by co-expression with the ER-marker mRFP-Sec61 β . FATP2 and FATP4 did neither co-localize with plasma membrane (ssGFPglycTMDpcx) nor peroxisomal (peroxisomal-mRFP1) marker proteins.

ACSL1 is one of the most extensively characterized proteins of the ACSL-family. Nevertheless its localization is still debatable [27, 29, 48, 49]. To specify the localization of FLAG-tagged ACSL1 we did a co-staining with the mitochondria-marker (mito-RFP) and found a co-localization in both HuH7 and HepG2 cell lines (figure 3). No overlap was found with plasma membrane (ssGFPglycTMDpcx), peroxisomes (peroxisomal-mRFP1), ER (mRFP-Sec61 β) or lipid droplets (adipophilin-mRFP1), where it has been previously described to be located as well [27].

Figure 4 shows that CD36 could be co-localized with PLAP, a marker for plasma membrane. The staining presented the complete size of the cell and typical branches on the cell surface. This localization is in line with previous results in COS cells [19]. No co-localization with mitochondria (mito-RFP), ER (mRFP-Sec61 β), lipid droplets (ADP-mRFP1) and peroxisomes (peroxisomal-mRFP1) could be detected.

Analysis of fatty acid uptake

To investigate the LCFA uptake in HuH7 and HepG2 cells, we transiently expressed FATP2, FATP4, ACSL1 and CD36 and incubated the cells with different FA substrates. Transcription of FATP4, FATP2, ACSL1, and CD36 was measured by efficiency corrected real-time PCR relative to β -actin to estimate the basal and overexpression rates. Transfection resulted in a several fold increase of mRNA expression: overexpression was 271-fold (ACSL1), 6777-fold (FATP2), 3165-fold (FATP4) and 181-fold (CD36) in HuH7 cells, respectively. To work with physiological fatty acids, we first used radio-labeled oleate in a 1:1 relation to BSA and incubated the cells for 60 sec with the mix.

All proteins significantly increased the oleate uptake in HuH7 (figure 5) as well as in HepG2 cells (data not shown) in comparison to GFP overexpression. GFP served as control and did not show any influence on LCFA uptake compared to non-transfected cells.

We also confirmed that overexpression of FATP2, FATP4 and ACSL1 leads to an increased acyl-CoA-synthetase activity in HuH7 cells (figure 6) and HepG2 cells (data not shown), when using oleate as substrate. CD36 had no influence on the ACS activity of the cells in comparison to the control.

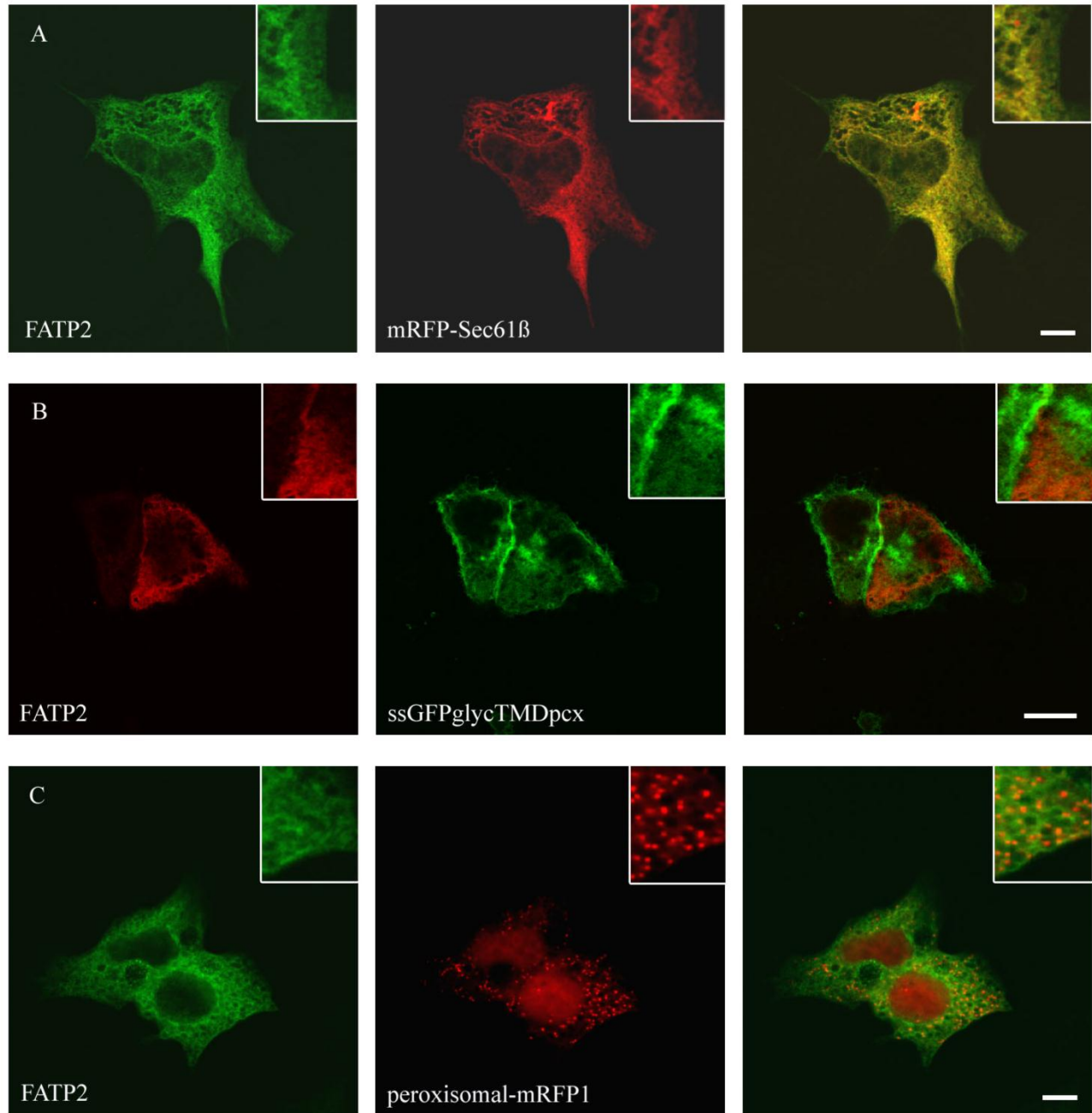


Figure 1. Localisation of FATP2 in HepG2 (A+C) and HuH7 (B) cells. The ER marker protein mRFP (red) was cotransfected. For FATP2-HA we used mouse anti-HA antibody from Santa Cruz and Cy2 fluorescently-labelled secondary antibody. FATP2 shows a reticular cellular structure representing ER membranes. There was no match with plasmamembrane marker (ssGFPglyc) (B) or peroxisomal marker proteins (peroxisomal-RFP) (C) in both cell lines. Bar: 10 μ m.

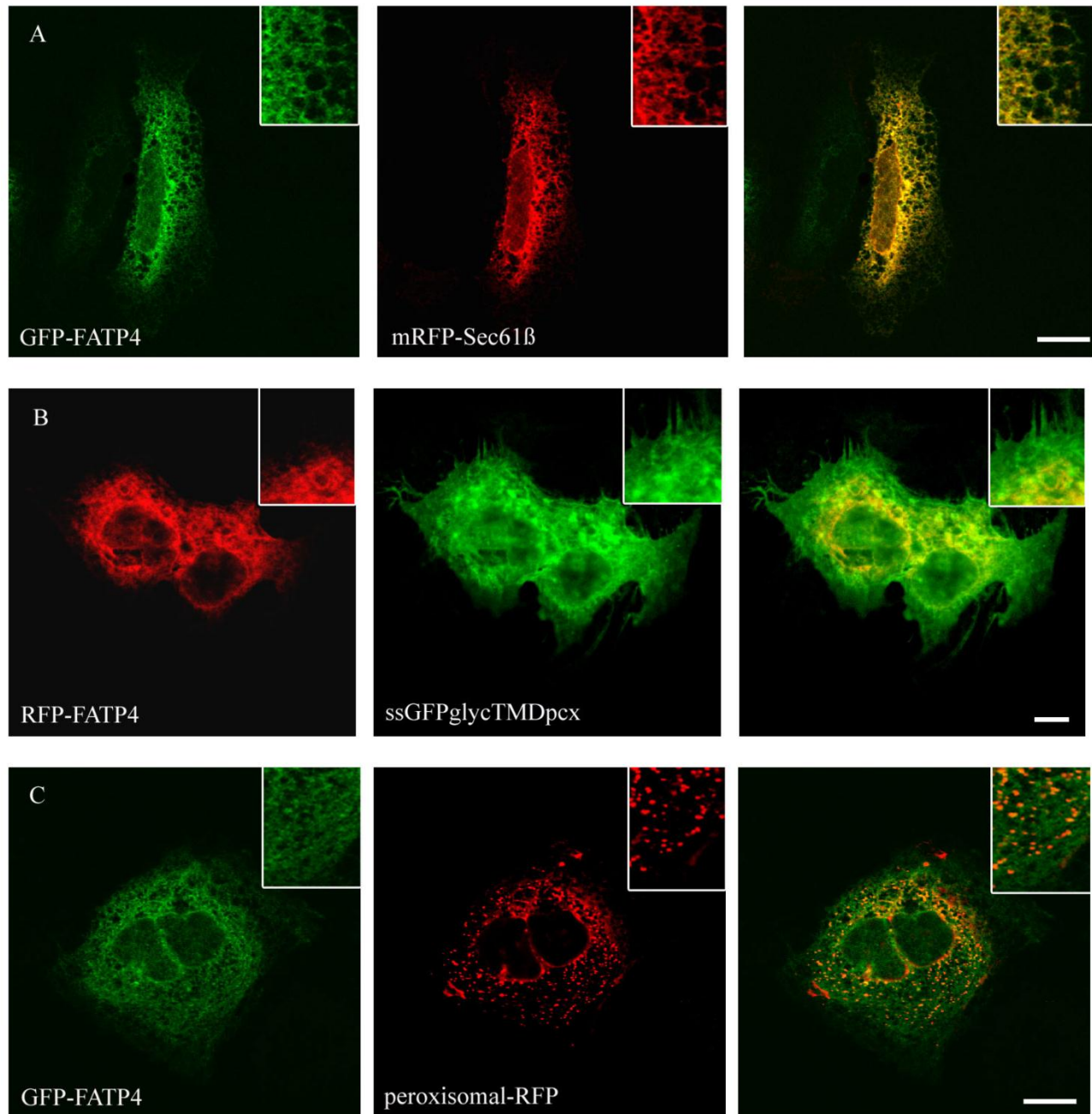


Figure 2. Localisation of FATP4 in HuH7 cells. GFP-FATP4 flags the same reticular structures as the ER marker protein mRFP (A). There was no concordance with plasmamembrane (ssGFPglyc) and RFP-FATP4 (C) or peroxisomal marker proteins (peroxisomal-RFP) and GFP-FATP4 (B). Results are representative for HepG2 cells as well. Bar: 10 μ m.

To compare FA uptake to the expression level of the individual protein at a single cell level, we performed FACS analysis as described [29]. FATP2, CD36 and ACSL1 were transiently overexpressed and incubated for a short time with the fluorescent long chain fatty acid B12. FATP4 was used as RFP-construct, FATP2, CD36 and ACSL1 were marked by immunofluorescence. In initial experi-

ments our cells were analysed by a confocal microscope and classified by visual characteristics. We could register an increased fluorescence level and thus increased B12 uptake upon overexpression of FATP2, FATP4 and ACSL1 compared to their non-expressing counterparts. Surprisingly, there was no significant uptake detectable upon overexpression with CD36 (figure 7).

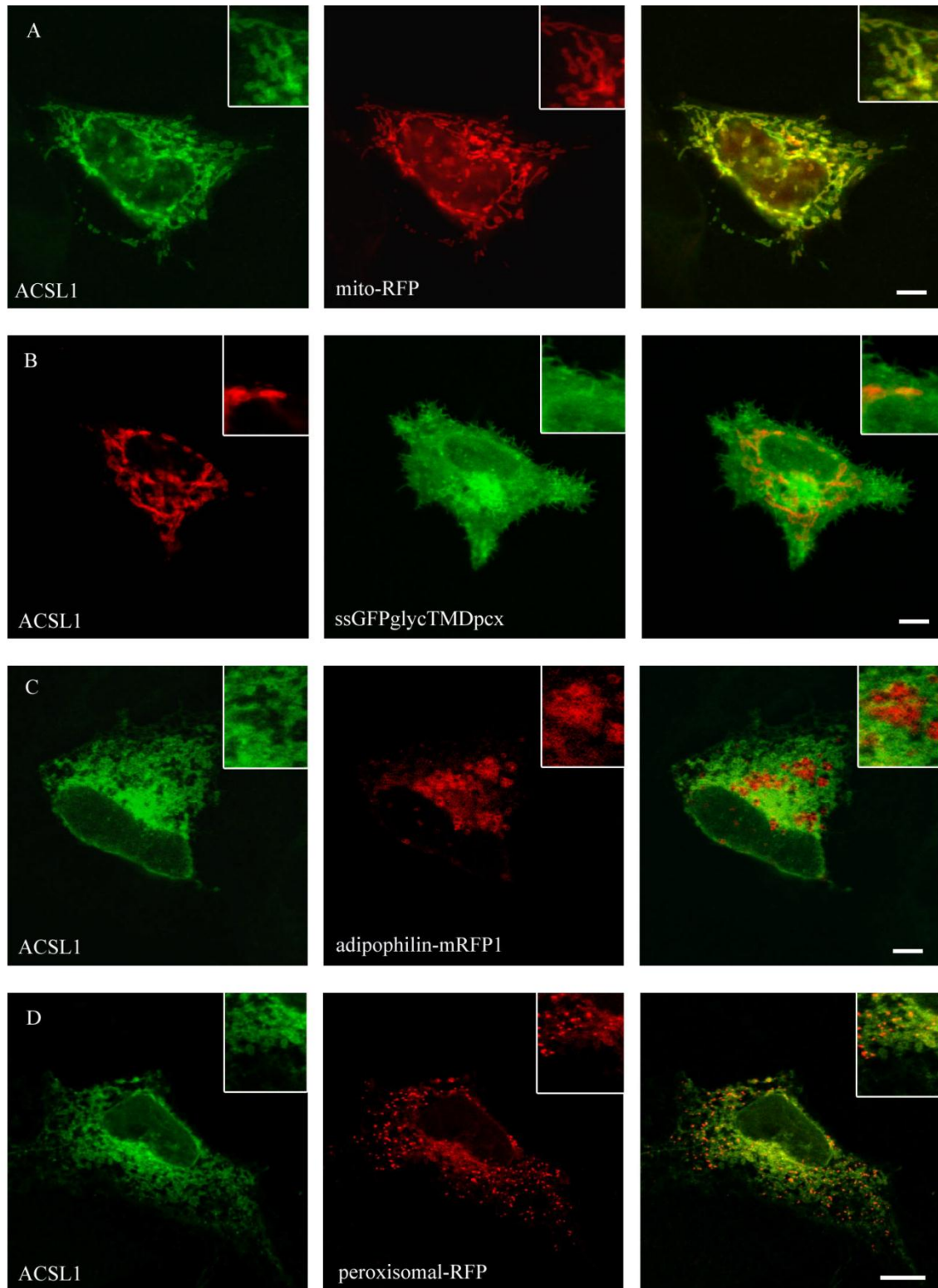


Figure 3. Localisation of ACSL1 in HepG2 cells. For ACSL1-FLAG we used mouse anti-FLAG antibody (Sigma) and Cy2 or Cy3 fluorescently-labelled secondary antibodies. HepG2 cells were cotransfected with mito-RFP, a marker for mitochondria and a plasmamembrane marker protein (ssGFPglyc). There was an overlap with the mitochondria staining (mito-RFP) (A) and no concordance with plasmamembrane staining (ssGFPglyc) (B). In addition ACSL1 had no overlap with lipid droplets (adipophilin-mRFP1) (C) or peroxisome (peroxisomal-RFP) marker proteins (D). Results are representative for HuH7 cells as well. Bar: 10 µm.

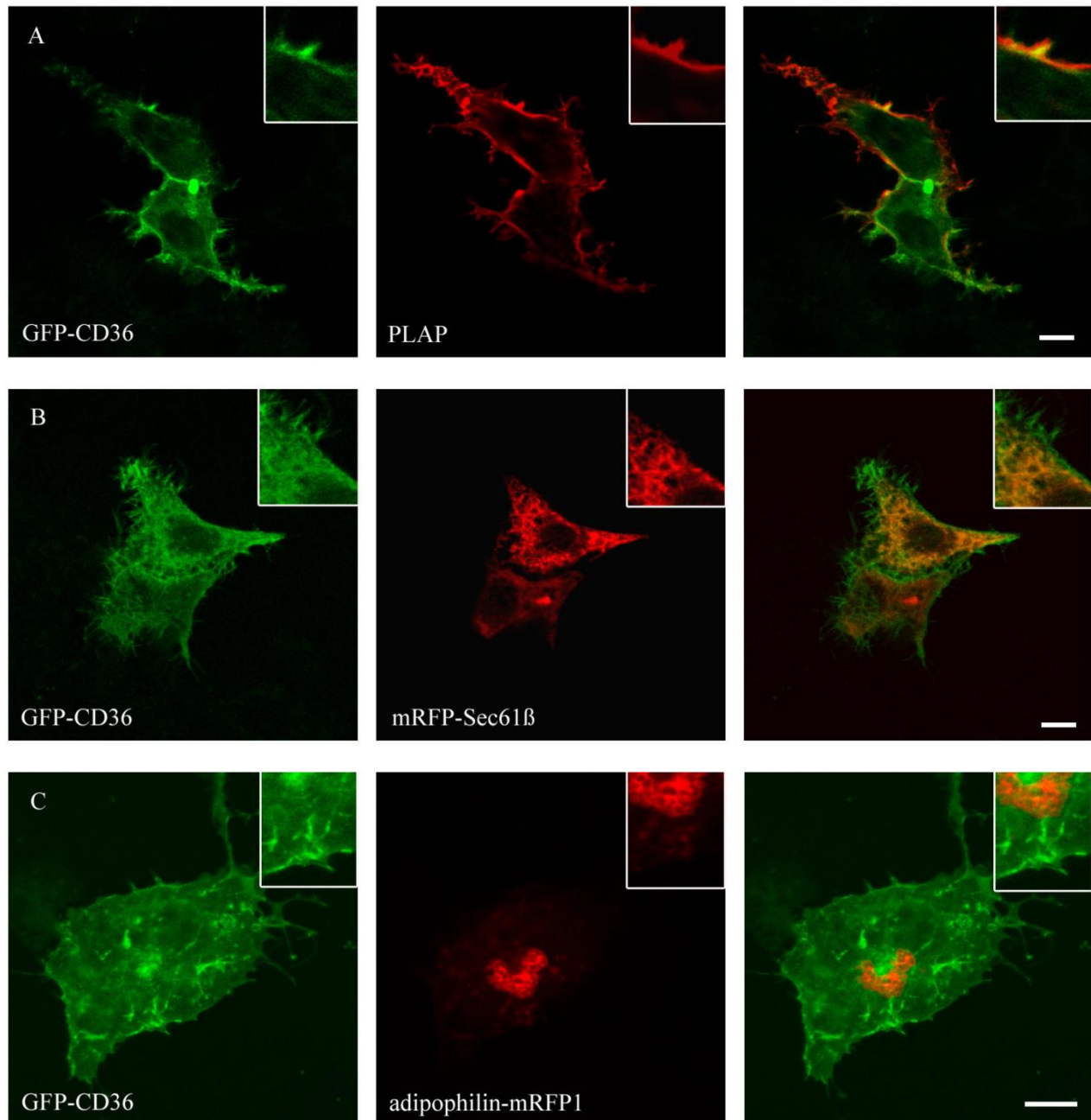


Figure 4. GFP-CD36 transiently cotransfected with PLAP in HepG2 cells and clustered with mouse anti PLAP. CD36 flags typical branches on the cell surface compatible with the plasma membrane (A). There was no matching of CD36 staining with ER (mRFP) (B) or lipid droplet (adipophilin-mRFP1) marker proteins (C). Results are representative for HuH7 cells as well. Bar: 10 μ m.

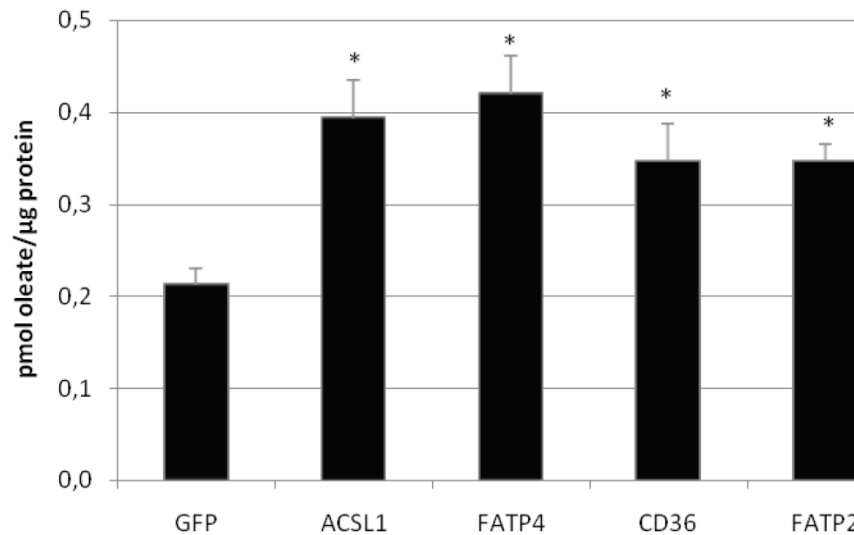


Figure 5. Oleate uptake of transiently transfected HuH7 cells after 60 sec of incubation with oleate-BSA mix (in pmol oleate/µg protein). Controls for endogenous oleate uptake were transfected with GFP plasmid. The protein overexpression resulted in a significant increase in the rate of [³H]oleate uptake within 60 sec ($p < 0.05$). Values are means \pm SD of three independent experiments.

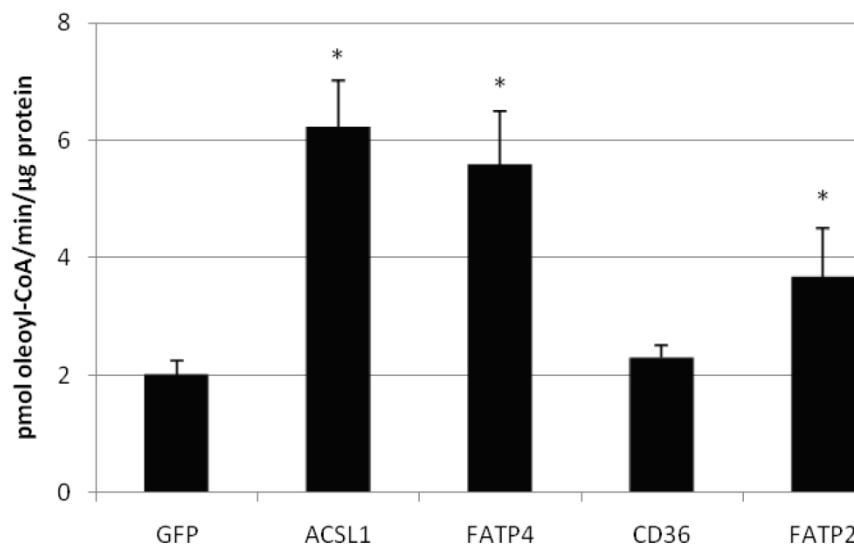
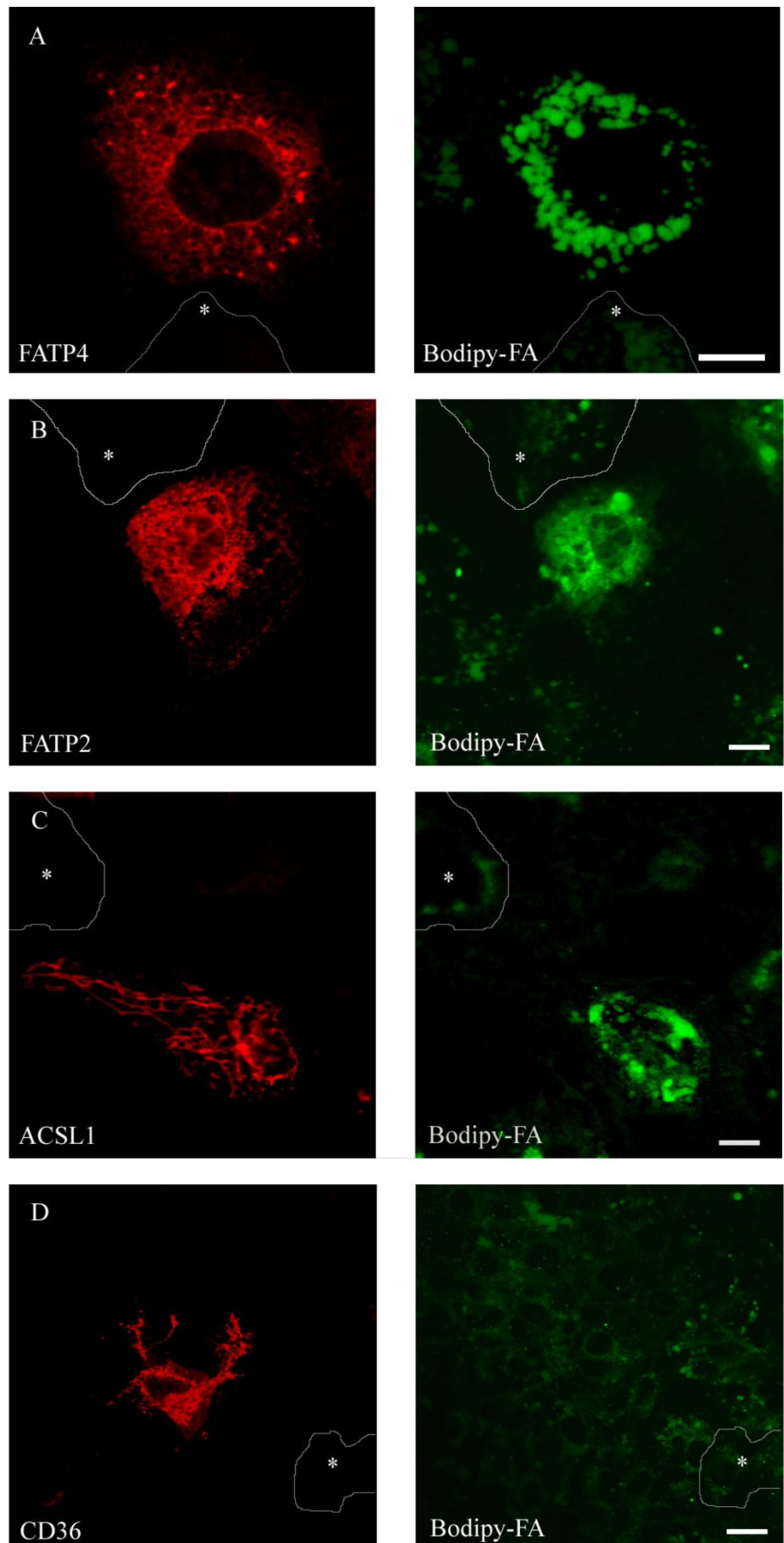


Figure 6. Acyl-CoA synthetase activity (in pmol oleoyl-CoA/minute/µg protein) determined from lysates of transiently transfected HepG2 cells. Controls for endogenous ACS activity were transfected with GFP plasmid. Values are means \pm SD of three independent experiments ($p < 0.05$).

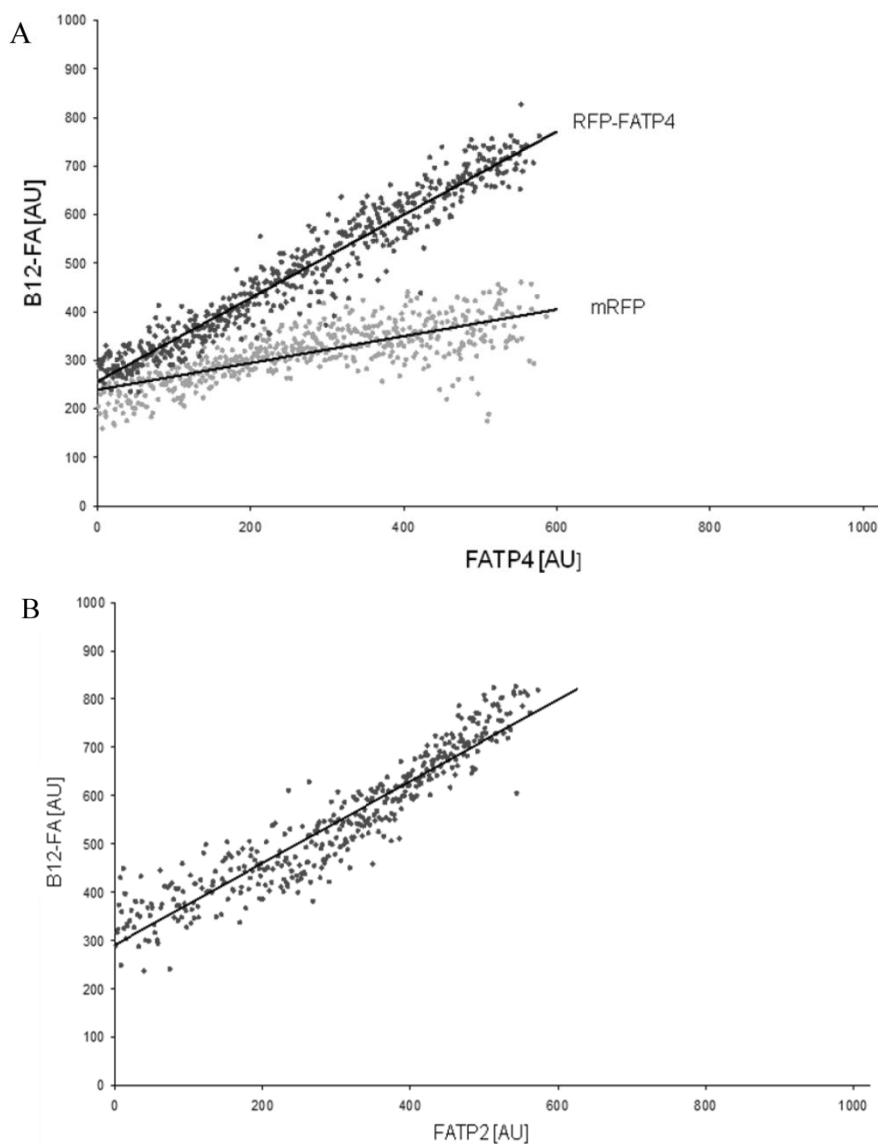
Figure 7. HuH7 cells with short time incubation of B12 fatty acid. Overexpression of RFP-FATP4, FATP2-HA and ACSL1-FLAG increased B12 uptake into the cell. B12 was accumulated and stored in lipid droplets. CD36-FLAG did not show any enhanced uptake of B12 compared to control cells.



To gain quantitative data a larger number of individual cells was analysed by FACS. The standard dot plot output of the FACS software confirmed that there is a higher B12 uptake with overexpression of FATP2, FATP4 and ACSL1, but not with CD36. Even though we could collect a large amount of cells with the FACS analysis, we could not correlate the B12 uptake with the level of expression of CD36 accurately.

To correlate individual transfected cells with their special fluorescent intensity of B12-FA that has been taken up, data were transformed by a spread-

sheet method (figure 8) [42]. Each data point in figure 8 contains all cells with the same fluorescence value of the transfected protein (equals the level of expression) and the average B12-FA fluorescence of these cells (equals the amount of FA taken up). Control cells were transfected with mRFP and pcDNA and show the endogenous B12 uptake of HuH7 cells. The calculated slopes (m) and the correlation coefficients (r) confirm a strong linear correlation between the level of protein expression of RFP-FATP4, FATP2-HA and ACSL1-FLAG and B12 uptake.



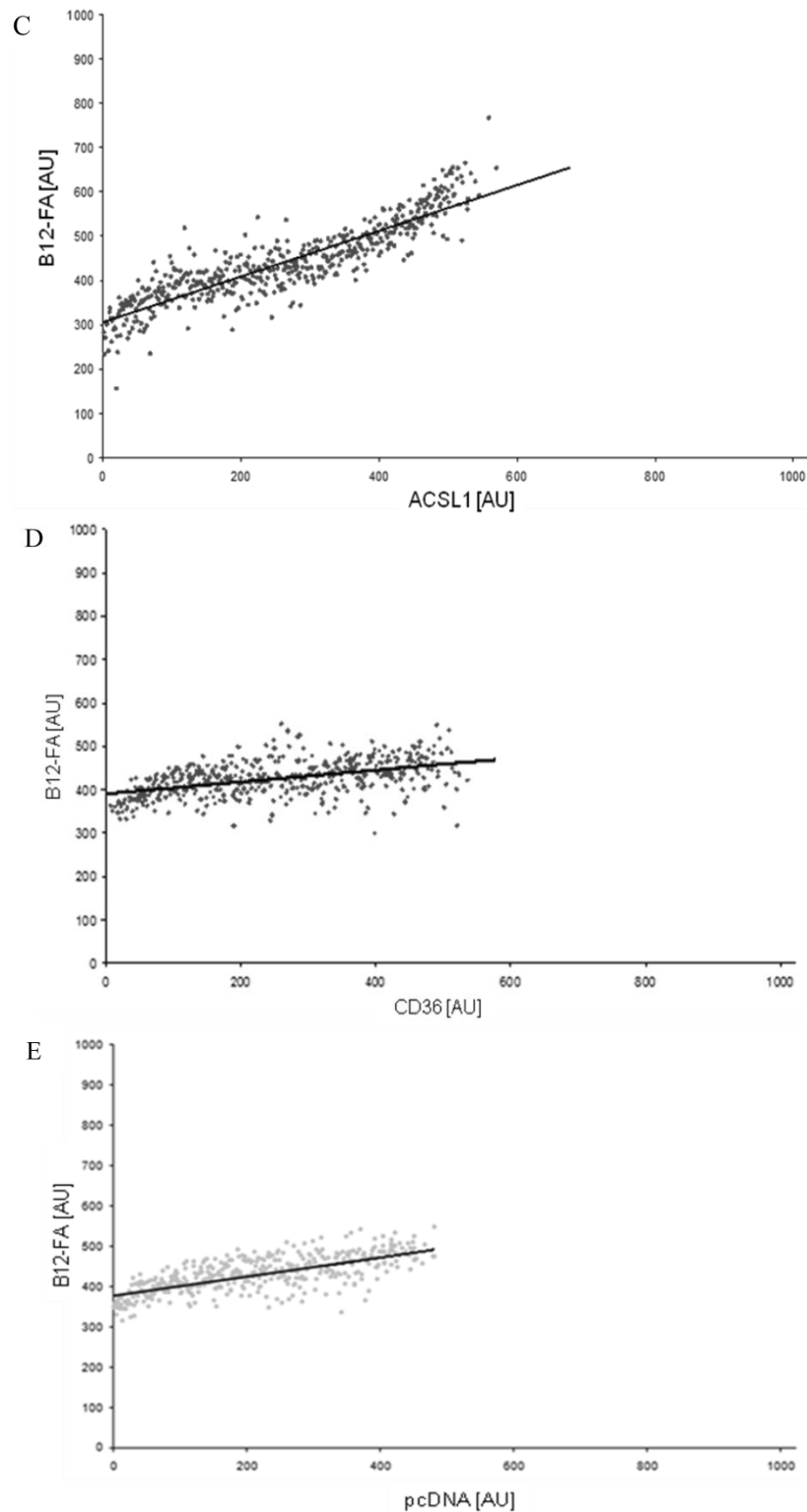


Figure 8. FACS analysis of fluorescent fatty acid uptake of transiently transfected HuH7 cells. These spreadsheets correlated the level of expression with the amount of fluorescent fatty acid uptake (B12-FA). (A) HuH7 cells transfected with RFP-FATP4. Control cells were transfected with red fluorescent mRFP protein. RFP-FATP4 ($r=0.73$) enhanced fatty acid uptake depending on its relative level of expression in comparison to control cells only expressing mRFP ($r=0.4$). The low increase of the fatty acid uptake of control cells ($r=0.4$) could be explained by endogenous FATP4 expression in HuH7 cells. FATP2-HA (B) ($r=0.92$) and ACSL1-FLAG (C) ($r=0.64$) overexpressing cells enhanced the uptake of fatty acids as well. The overexpression of CD36-FLAG (D) ($r=0.2$) did not significantly correlate with the uptake of fatty acids. Control cells were transfected with pcDNA3 (E) ($r=0.18$). The pictured diagrams are representative for three independent experiments.

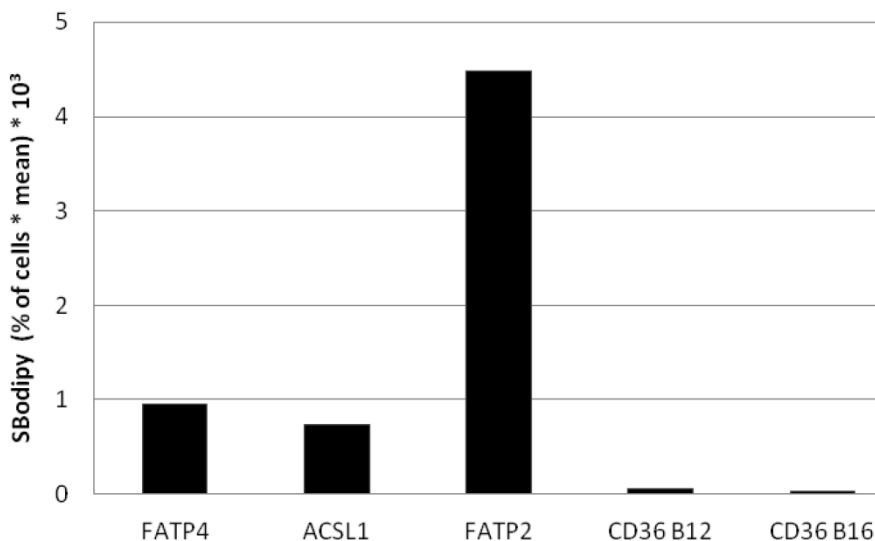


Figure 9. Bodipy fatty acid uptake of RFP-FATP4, CD36-FLAG, FATP2-HA and ACSLI-FLAG transfected HuH7 cells in arbitrary units based on FACS analyses. Bodipy fatty acid uptake was correlated to the protein expression level of transfected protein on a single cell basis. The gated overexpressing cells (in % of all cells gated) were multiplied with the corresponding overall mean of the fluorescence signal derived from the Bodipy fatty acid (S_{Bodipy}). HuH7 Cells transfected with pcDNA3 were used as control and as background range and were set as base level of the x-axis. Uptake was performed with B12 fatty acid in experiments with FATP4, FATP2, ACSLI and CD36 (CD36 B12) or with B16 (CD36 B16). The pictured diagram is representative for three independent experiments.

To find out, which one of the investigated proteins causes the quantitative highest FA uptake, we compared Bodipy fatty acid uptake to the fluorescent level of the overexpressed candidate protein on a single cell basis. The percentage of gated cells was multiplied with the mean of the fluorescence signal derived from B12. Presenting this data in arbitrary units shows that FATP2 overexpressing cells seem to have the quantitative highest uptake of fluorescent fatty acid compared to their expression levels (figure 9). This means less FATP2 within one cell is necessary to increase FA uptake to a similar level compared to other candidate proteins.

Again we could not find any increased uptake of B12-FA when HuH7 cells were transfected with CD36. In contrast to studies of Koonen et al. [50] even with another ligand (Bodipy-C16) there was no difference between the uptake of untransfected cells and cells with CD36 overexpression (figure 9). This likely indicates that Bodipy FAs – in opposite to [3H]oleate – are no ligands for CD36.

DISCUSSION

Translocation of long chain fatty acids at the plasma membrane is achieved by a complex interplay of several co-existing mechanisms [1, 3]. Members of

the FATP family have been previously proposed to be the long sought transport proteins that facilitate LCFA uptake at the plasma membrane. However, this is still a matter of debate [1-4, 6, 8]. Recent data suggest that at least FATP4 is an intracellular enzyme localized in the ER [29, 47]. In our studies we could localize FATP2 and FATP4 only in the ER of HuH7 and HepG2 cells. This confirms other results which found FATP4 in the ER of mouse small intestine, COS, MDCK, 3T3-L1 adipocytes and Vero cells [29]. In accordance to Steinberg et al. we could find FATP2 in the ER of hepatoma cells [46]. Other studies located FATP2 at the plasma membrane of hepatocytes [34] or in fibroblast peroxisomes promoting FA converting via β -oxidation [45]. We could not affirm these findings in our cell lines. The reason for this difference remains unclear, but it could be due to different methods of overexpression, materials and cells used for analysis. Consequently, in Huh7 as well as in HepG2 cells, FATP4 as well as FATP2 are no transport proteins directly facilitating LCFA transport at the plasma membrane. In fact, they are located intracellular and are involved in FA metabolism (probably production of phospholipids and triglycerides) upon their acyl-CoA activity. They more likely increase LCFA uptake indirectly by metabolic trapping, which means

that long chain fatty acids are activated and then metabolized. A decreased intracellular free LCFA concentration would drive more LCFAs into the cell [2, 8].

Acyl-CoAs are involved in both anabolic and catabolic pathways. The disturbance of these pathways is linked to hepatic steatosis, hyperlipidemia and insulin resistance [51]. Increased LCFA uptake could also be correlated to the ACSL1 activity [29] and accumulation of triglycerides could be shown upon overexpression in HepG2 cells [52]. Therefore we also analyzed the localization and effect of ACSL1 in our hepatoma cell models. In contrast to previous results on primary rat hepatocytes where ACSL1 was located in ER [49], we could point out its localization in mitochondria of HuH7 and HepG2 cells. This would be in line with their potential role in mice adipocytes [53]. In the majority of cases the localization in hepatocytes has been studied when ACSL1 was overexpressed, therefore ER localization might also be a phenomenon of different levels of overexpression. Another explanation might be a different function of ACSL1 depending on the cell type or tissue.

Our studies affirm, that FA uptake into hepatoma cells can be regulated by enzymes. Accordingly, we could identify an increased oleate uptake in addition to an elevated ACS activity for ACSL1, FATP2 and FATP4 after overexpression. We believe that increased enzyme activity could diminish the intracellular pool of free LCFA and create a concentration gradient of free LCFA in the intra- and extracellular domain. For an increased LCFA uptake ACS have therefore not to be localized at the plasma membrane as found in our results.

As we could demonstrate an increased [3H]oleate uptake upon FATP2, FATP4, ACSL1 and CD36 overexpression compared to control cells, we have to limitate, that expression levels by transient transfection are varying from cell to cell in these experiments. As the Bradford assay measures the total protein level of each sample, we could not draw conclusions to the amount of overexpressed protein reliably. Accordingly, these data as well as the data of ACS activity should not be considered quantitatively but qualitatively.

For this reason we performed fatty acid uptake assays on a single cell level via FACS analyses and spreadsheet calculations. We could affirm that at least FATP2, FATP4 and ACSL1 have significant influence on the level of LCFA uptake depending on their expression levels. Therefore they can be important targets for the treatment of metabolic liver diseases as a dose dependent effect of inhibition seems likely. Interestingly, targeting FATP2 and FATP4 have already

become more attractive as potent small molecule inhibitors have been identified [33, 54].

In our FACS analysis we could identify FATP2, the most expressed FATP in liver cells [30], to have the highest influence on LCFA uptake in correlation to its protein expression level in hepatoma cells. Therefore the treatment of nonalcoholic fatty liver disease (NAFLD) or NASH by inhibiting the uptake of FA into the liver cell could be theoretically influenced by blocking especially FATP2 function. Interestingly, actual data show that liver specific inhibition of FATP2 expression in vivo resulted in a reduced hepatosteatosis with improving glucose and insulin levels [34]. In addition, recent results from an high throughput screen on HepG2 and CaCo-2 cells demonstrate that FA transport can be inhibited in a FATP-specific manner without causing cellular toxicity [33]. This makes FATP2 to a target of high interest for further metabolic strategies.

When overexpressed in cultured fibroblasts CD36 increases LCFA uptake [55]. Moreover, CD36 knockout mice show reduced uptake of oleate in isolated adipocytes [56]. In HuH7 and HepG2 cells we could increase [3H]oleate uptake by transient overexpression of CD36 significantly. In FACS analyses an enlarged uptake of B12 FA was missed. Even with B16FA, previously been shown to be a CD36 ligand [50], there was not a significant gain of FA uptake (figure 9). The differences between [3H]oleate and Bodipy fatty acid could probably be explained by special substrate specificities of CD36.

We could show that CD36 is located in the plasmamembrane of HuH7 and HepG2 cells. This strengthens results from other studies that locate CD36 in raft and non raft plasma membrane domains [19, 20] and suggests a significant role of CD36 in the uptake mechanism of FA in hepatoma cells. As CD36 has no ACS activity, it possibly increases FA uptake by accumulating FA at the plasma membrane. Recent data show that CD36 is indeed increased in liver of morbidly obese patients and correlated to free FA levels [22]. It has also been shown that downregulation of CD36 can prevent steatohepatitis in hyperlipidemic mice [57]. This way CD36 is also a target for the treatment of NAFLD or NASH.

In conclusion, we studied four different proteins that can promote FA uptake into hepatoma cells depending on their expression levels. An attractive therapeutic candidate seems to be FATP2 as it had the strongest influence on fatty acid uptake in our experiments. It is highly expressed in liver tissue and an effective selective inhibition with small molecules has already been shown [33]. It will be interesting to see, if

this concept could be really integrated into therapeutic strategies.

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CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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