# Stable transfection of fatty acid translocase (CD36) in a rat heart muscle cell line (H9c2)

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**Abstract Fatty acid translocase (FAT/CD36) is a membrane protein putatively involved in the transmembrane transport of long-chain fatty acids. We tested the hypothesis that expression of this protein in H9c2, a rat heart cell line normally not expressing FAT, would increase cellular palmitate uptake. We were able to stably transfect H9c2 cells with FAT, yielding 15 cell lines showing varying levels of FAT expression. The uptake and metabolism of palmitate was first studied in the non-transfected H9c2 cells and in two FAT-transfected cell lines. In each case, uptake of palmitate was found to be linear in time for at least 30 min and the uptake rate was saturable with increasing palmitate concentrations. Using conditions under which the maximal capacity of intracellular palmitate handling was not fully utilized, we tested 7 out of 15 FAT-transfected cell lines with varying FAT expression levels. No significant correlation was found between the level of FAT expression and the rate of palmitate uptake. In conclusion, we found that palmitate uptake by H9c2 cells occurs mainly by passive diffusion. Fatty acid translocase (FAT) transfection did not significantly increase the palmitate uptake rate, raising the possibility that H9c2 cells lack a protein (or set of proteins) that acts as an obligatory partner of FAT in long-chain fatty acid transport from the extracellular compartment to the cytoplasm.—**Van Nieuwenhoven, F. A., J. J. F. P. Luiken, Y. F. De Jong, P. A. Grimaldi, G. J. Van der Vusse, and J. F. C. Glatz. **Stable transfection of fatty acid translocase (CD36) in a rat heart muscle line (H9c2). *J. Lipid Res.* 1998.** 39: **2039–2047.**

**Supplementary key words** palmitate uptake • long-chain fatty acids • protein-mediated fatty acid uptake

The precise mechanism of the transport of long-chain fatty acids across the sarcolemma of cardiac myocytes is a matter of considerable debate (1–3). Some investigators favor passive diffusion of long-chain fatty acids through the phospholipid bilayer as the main transfer process (4–6), while others reported data suggesting that the sarcolemmal transport of long-chain fatty acids is mediated by membrane-associated proteins (7, 8). Recently obtained evidence indicated that the bulk of palmitate uptake by isolated cardiac myocytes is mediated by membrane-associated proteins, and that only a minor part of palmitate is taken up by passive diffusion (9). One of the proteins hypothesized to be involved in trans-sarcolemmal long-chain fatty acid transport is fatty acid translocase (FAT) (10, 11). This protein, also known as CD36, was identified in adipocyte membranes by covalent labeling with sulfo-*N*-succinimidyl oleate (SSO). Binding of SSO to FAT resulted in a ca. 70% inhibition of long-chain fatty acid uptake (12). Moreover, isolated perfused rat hearts showed a decreased palmitate uptake after labeling of FAT with sulfo-*N*-succinimidyl palmitate (13). Recently, expression of FAT in fibroblast cells normally lacking this protein (Ob17PY cells) was shown to increase cellular uptake of oleate and palmitate (14). In addition, FAT was found to bind several different long-chain fatty acids reversibly and with high affinity (15). Besides its putative role in long-chain fatty acid uptake, FAT/CD36 possesses a number of other hypothesized functions, as recently reviewed (16, 17).

The objective of the present study was to investigate palmitate uptake and metabolism in the clonal rat muscle cell line H9c2 and in FAT-transfected H9c2 cells. This cell line is derived from embryonic rat heart tissue and initially was described to have properties of skeletal muscle cells (18). However, subsequent studies by other investigators showed that H9c2 cells also exhibit specific properties of cardiac myocytes, such as the expression of the cardiac isoform of L-type calcium channel (19–21) and the tissue-specific splicing protein SmN (22). It was concluded that this cell line has both cardiac and skeletal muscle characteristics (21). As H9c2 cells normally do not express FAT as found by Northern and Western blot analyses (unpublished observations), we reasoned that this cell line forms a suitable model system to study FAT function in muscle

Abbreviations: FAT, fatty acid translocase; FATP, fatty acid-transport protein; FABPpm, plasmalemmal fatty acid-binding protein; SSO, sulfo-n-succinimidyl oleate; DIDS, diisothiocyano-stilbene-disulfonic acid; BSA, bovine serum albumin.

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cells. It is hypothesized that palmitate uptake is correlated with FAT expression and therefore will be increased in H9c2 cells upon transfection with this protein. The results show, however, that palmitate uptake in H9c2 cells is most likely not protein-mediated and that in FAT-transfected H9c2 cells the uptake rate of palmitate is not uniquely correlated with the degree of FAT expression.

## MATERIALS AND METHODS

### Cell culture

H9c2(2-1) cells (CRL1446) were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and were used between passages 20 to 30. These cells, which will be designated H9c2 throughout this paper, were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Life Technologies, Gaithersburg, MD) supplemented with 9% (vol/vol) fetal calf serum (FCS, SEBAK, Aidenbach, Germany), further called standard medium. Cells were plated at a density of about $10^4$ cells/$cm^2$ in 100 mm dishes (Falcon, Becton Dickinson, Plymouth, UK) and were grown under 5% $CO_2$ in water-saturated air.

### Transfection and subsequent selection of stably transfected cells

Three 100 × 20 mm Falcon dishes of 50% confluent H9c2 cells (passage 23) each were transfected with a mixture of the plasmids pSG5-FAT (10 μg) and pMAMneo (0.5 μg) (14). pSG5-FAT was constructed by inserting the coding region of FAT (about 1.4 kb) into the BamH1 site of the eukaryotic expression vector pSG5 (Stratagene, La Jolla, CA) as was described earlier (14). Cotransfection of both plasmids into the H9c2 cells was performed using the lipocarrier DOTAP (Boehringer, Mannheim, Germany), exactly according to the manufacturer's instructions. The transfection mix was added to the cells at the end of the day and the transfection was carried out overnight (incubation time was ca. 15 h). Thereafter, the transfection mix was removed and fresh standard medium was added to the cells. At the end of the same day the cells were trypsinized (0.25% trypsin from Gibco, in PBS), replated in three different dilutions (2×, 4× and 8×), and selection medium was added to the cells. Selection medium consisted of standard medium supplemented with 400 mg/l geneticin (G418, Gibco). Untransfected H9c2 cells (control) were treated identically, so as to check the efficiency of the selection. Selection was carried out for 4 weeks and control cells all died within 3 weeks. On the dishes used for transfection, cells were grown as colonies, and several colonies were selected and isolated. Twenty-six colonies were isolated, of which 15 could be maintained as cell lines. These cell lines were propagated until at least 10 aliquots of each cell line had been stored in liquid nitrogen. After selection, cells were cultured in maintenance medium, which consisted of standard medium supplemented with 200 mg/l geneticin.

### Southern blotting

Incorporation of FAT cDNA into the H9c2 genome was studied by Southern blot analysis. Genomic DNA was isolated from control and transfected cells using DNAzol reagent (Gibco). Thereafter, 10 μg DNA from control H9c2 and from the geneticin resistant cell lines were incubated with BamH1. Cell lines that incorporated the pSG5-FAT plasmid should yield a 1.4 kb FAT cDNA fragment. The DNA fragments were separated on a 1% agarose gel and blotted onto a Hybond N+ nylon membrane (Amersham, Little Chalfont, UK). DNA was fixed by drying for 15 min at 80°C followed by crosslinking under ultraviolet light (0.4 J/$cm^2$). Thereafter, the blot was probed with the cDNA of FAT that was labelled using the Radprime DNA Labelling System from Gibco. Filters were prehybridized for 2 h and hybridized overnight at 56°C. Aspecific hybridization was removed by several washing steps at 56°C with decreasing salt concentrations. The final washing step was performed with a buffer consisting of 0.1 × SSC (saline sodium citrate, 0.15 m NaCl, 0.017 m $Na_3C_6H_5O_7$, pH 7.0) containing 0.1% SDS.

### Northern blotting

To check mRNA levels of FAT in the cell lines obtained after selection, 5 μg of total RNA (isolated using Trizol reagent from Gibco) was separated by electrophoresis and blotted on a nylon membrane (Hybond-N, 0.45 micron, Amersham) as described earlier (11). RNA was fixed by drying for 15 min at 80°C followed by crosslinking under ultraviolet light (0.4 J/$cm^2$). Subsequently, RNA was stained with methylene blue and the 28S and 18S ribosomal bands were quantified using a hand scanner (Primax, Zeist, the Netherlands) and the software programs Finishing-Touch (U-Lead Systems, Taipei, Taiwan) and ImageQuant (Molecular Dymanics, Sunnyvale, CA). RNA isolation, electrophoresis, and transfer to Hybond-N was successful as judged by the staining of the RNA with methylene blue. Quantification of the ribosomal RNA bands revealed that the 28S signal was stronger than the 18S signal in all cases, indicating that no major RNA degradation had occurred. The variation in amount of RNA present on the different lanes of the blot was about 25%. The results of this quantification were used to normalize the results from subsequent labeling with FAT cDNA on the amount of RNA present on the blot. Thereafter, the blots were probed with the cDNA of FAT which was labeled using the Radprime DNA Labelling System from Gibco. Filters were prehybridized for 1 h and hybridized overnight, and non-specific binding was removed in several washing steps with increasing temperature and decreasing salt concentration. The final washing step consisted of 30 min incubation in 0.1 × SSC containing 0.1% SDS at 56°C. Filters were exposed to X-ray film and to imaging screens for scanning and quantification (Phosphor Imager, ImageQuant; Molecular Dynamics).

### Western blotting

A monoclonal antibody raised against human CD36 (MO25) was kindly provided by Dr. D. E. Greenwalt (Human Genome Sciences, Rockville, MD). Purified recombinant rat FAT, expressed in insect cells was kindly provided by Dr. N. A. Abumrad (State University of New York at Stony Brook, NY). Tissue and cells were electrophorized and blotted onto nitrocellulose (pore size 0.45 μm) using the Mini-Protean II Electrophoresis System from Bio-Rad (Hercules, CA). To avoid non-specific binding of antibodies in subsequent steps, the blot was incubated for 1 h in PBS containing 5% BSA. The blot then was incubated for 1 h with a 1:2000 dilution of the MO25 antibody in PBS containing 3% non-fat dry milk and 0.05% Tween-20. Thereafter, the blots were washed three times for 5 min with PBS/Tween-20 and incubated for 1 h with a 1:2000 dilution of peroxidase-conjugated rabbit anti-mouse IgG, P0161 (DAKO, Glostrup, Denmark) in PBS/Tween-20 containing 3% non-fat dry milk. After eight washes (5 min each with PBS/Tween-20) the detection of peroxidase activity was performed using the ECL western blotting analysis system of Amersham. Semiquantitive results of FAT protein expression were determined using the handscanner and the software program Finishing-Touch as described for the Northern blotting procedure.

### Immunofluorescent labeling of FAT

A monoclonal antibody (131.4) raised against human CD36 was kindly provided by Dr. N. N. Tandon (Otsuka Pharmaceutical

Co., Rockville, MD) and was used as primary antibody in the immunofluorescence experiments. An FITC-labeled rabbit anti-mouse IgG (DAKO) was used as secondary antibody. Cells were fixed by methanol/acetone or by 1% paraformaldehyde. In addition, living cells (both attached or trypsinized) were incubated with the antibodies in the presence of azide (0.1%) to prevent incorporation of antibodies so as to verify the localization of FAT on the plasma membrane. Incubation with the primary antibody was performed in PBS/BSA (1%) for approximately 1 h at room temperature. The second antibody was incubated at room temperature for about 30 min. After the labeling procedure, cells were mounted in Tris-buffered glycerol containing 1,4-diazabicyclo-[2,2,2]-octane (DABCO) and 4′,6-diamidino-2-phenylindole (DAPI).

### Palmitate uptake studies

Studies on palmitate uptake were performed with the FAT-transfected cell lines between passages 3 and 10 after transfection. Untransfected H9c2 cells were studied between passages 20 and 30. Cells were washed twice with PBS and subsequently incubated with PBS containing 0.05% trypsin and 0.2 mm EDTA (mild trypsinization) for approximately 3–5 min. The cells detached from the culture dish and were suspended in Krebs-Henseleit bicarbonate medium (pH 7.4) supplemented with 11 mm glucose, 0.4 mm taurine, 1 mm $CaCl_2$ and 0.3 mm BSA (Sigma, A7906) (medium A). The fatty acid content of this BSA batch was measured gaschromatographically (23) and found to be 0.09 mole per mole of BSA, of which one third was palmitate, while the remaining part was mainly formed by oleate, stearate, and linoleate.

For the measurement of palmitate uptake, 1.5 ml of cell suspension (5–10 mg wet mass/ml) was preincubated in glass vials at 37°C under carbogen (95% $O_2$ and 5% $CO_2$) for 5 min. Subsequently, 0.5 ml of medium A containing variable levels of $^{14}$C-labelled palmitate was added. The preparation of the palmitate–BSA complexes was performed exactly as described by Luiken et al. (9). After the desired incubation time, the uptake was stopped by pipetting 1 ml of the incubation mixture to 9 ml of ice-cold stop-solution, which was medium A, without glucose, containing 15 μM BSA and 0.2 mm phloretin, as described earlier (7, 9). The combination of these agents removes surface-bound palmitate from the cells, while completely blocking further efflux of palmitate from the cells (7, 9). The cells were centrifuged for 6 min at 150 *g* at 4°C and washed with ice-cold stop-solution and this step was repeated twice. The cell pellet then was transferred to 5 ml of scintillation fluid (formula 989, Packard, Meriden, CT) and the radioactivity was measured.

All incubations were performed in duplicate. Wet mass of cells was determined in duplicate by weighing the cell pellet after centrifugation at 200 *g* for 10 min. The protein content of the cell lines was determined in separate experiments using the BCA method (Pierce, Rockford, IL). The protein content of the H9c2 cells and of the FAT-transfected cell lines amounted to approximately 60 mg/g wet mass of cells. (The protein content of H9c2 was 59 ± 10, that of 1A3 was 61 ± 8, and that of 3B3 was 59 ± 8, means ± SD, for 10, 9, and 6 measurements, respectively).

To test whether the mild trypsinization, used to dissociate the cells from the culture dish, affected the palmitate uptake of the cell lines, a number of experiments were performed with attached cells in petri dishes. These experiments showed that palmitate uptake was similar in suspended and attached cells (data not shown).

### Statistical analysis

Results of the palmitate uptake studies are presented as means ± SD and were analyzed for statistically significant differences using the Mann-Whitney method. Kinetic data from palmitate uptake experiments were studied according to Zivin and Waud (24), using the Eadie-Hofstee plot to calculate $V_{max}$ and $K_m$. The correlation between FAT-expression and palmitate uptake rate was tested using the Spearman method. A $P$-value $< 0.05$ was considered significant.

## RESULTS

### Transfection of H9c2, and selection of cell lines

Upon transfection of the H9c2 cells with a mix of the plasmids pSG5-FAT and pMAMneo, 26 colonies were selected, isolated, and cultured in selection medium. Fifteen of these colonies survived the selection and could be maintained as cell lines. Except for one of the cell lines (designated 1A1), the cell lines showed similar morphologies and growth rates. The 1A1 cell line grew faster and the cells were round instead of spindle-shaped. Southern blot analysis showed that this cell line incorporated a significantly larger amount of FAT cDNA into its genome than did the other cell lines (data not shown). The cell lines were cultured for several passages and a number of batches of each cell line were stored in liquid nitrogen. In addition, cells were harvested for RNA, DNA, and protein analyses.

### Expression of FAT in transfected cell lines

FAT cDNA had been incorporated into the genome of 9 cell lines while no signal was found in 4 cell lines, i.e., 3B1, 3B2, 3B3, and 3B4 (2 cell lines were not included in the Southern blot analysis). Southern blot analysis using pMAMneo as probe indicated incorporation of this plasmid in the genome of all transfected cell lines tested (data not shown).

Northern blot analysis was performed with total RNA from the 15 cell lines obtained after FAT-transfection, and from rat heart, which served as control RNA for FAT expression. The results obtained for 13 of the 15 FAT-transfected cell lines and rat heart RNA (control) using FAT cDNA as probe are shown in **Fig. 1**. A single strong signal

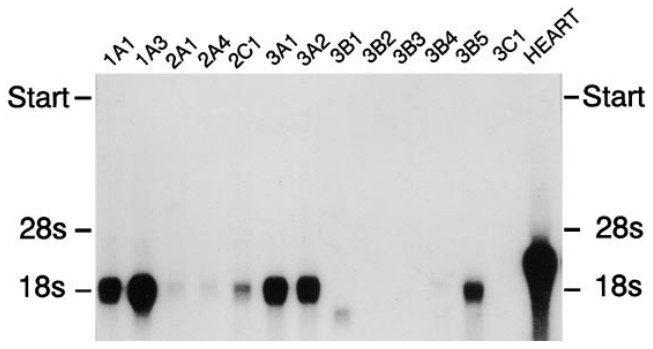


**Fig. 1.** Northern blot analysis of RNA isolated from 13 FAT-transfected cell lines (1A1 through 3C1), using FAT cDNA as probe. Rat heart RNA was included as control. 28S and 18S indicate the position of the signals for these ribosomal RNAs upon staining with methylene blue.

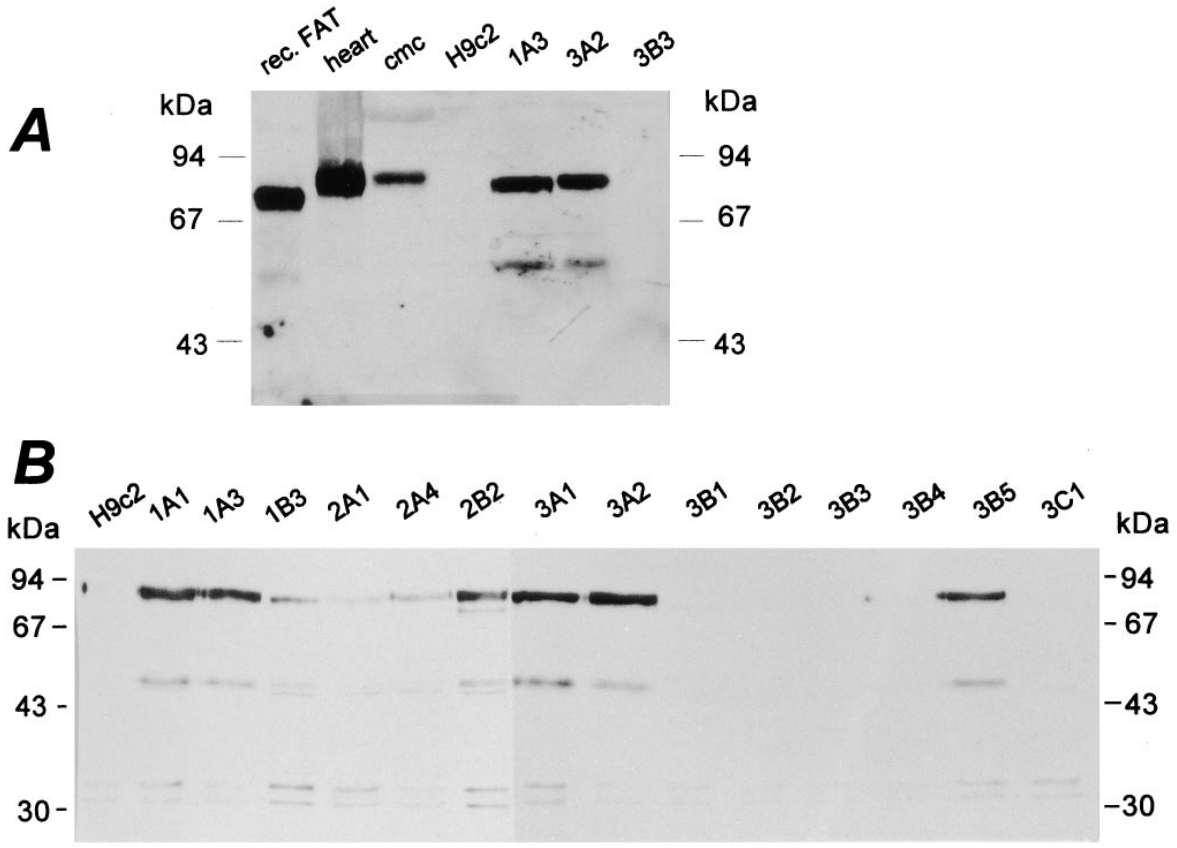


**Fig. 2.** Western blot analysis using monoclonal MO25, raised against human CD36, as primary antibody. Panel A: purified recombinant FAT, rat heart, isolated cardiac myocytes (CMC), H9c2, 1A3, 3A2, and 3B3 cells. Panel B: H9c2 and 14 FAT-transfected cell lines (1A1 through 3C1).

for FAT is seen in the lane of rat heart in between the 28S and 18S signal, which is in accordance with a molecular mass of about 2.9 kb, as was found in earlier studies (10, 11). Several cell lines showed a single strong signal for FAT, but in each case this is found at a lower molecular mass (1.9 kb). Differences between the mRNAs from the endogenous gene and the transfected cDNA with respect to the lengths of the 5′ and 3′ untranslated regions and the poly A tail most likely are responsible for this finding.

FAT protein expression was studied using Western blotting. The result of Western blot analysis for 14 of the 15 cell lines is shown in **Fig. 2**. For comparison, pure recombinant FAT, rat heart, and isolated cardiac myocytes (CMC) were also analyzed. Recombinant FAT shows a signal at a lower molecular mass than the signal found in rat heart or CMC, probably due to differences in glycosylation of FAT. A distinct signal can be seen at approximately 88 kDa in rat heart and CMC, and in some of the cell lines, while control H9c2 cells are negative. In addition, cell lines with high FAT expression show an extra band between 43 kDa and 67 kDa, which might correspond to the non-glycosylated FAT protein (about 53 kDa in molecular mass). Both the signals from the Northern blots and those from the Western blots were quantified as described in the Materials and Methods section. A scatter diagram with the protein levels and the mRNA levels of FAT in the 15 FAT-transfected cell lines shows that for cells expressing moderate amounts of FAT there is a linear relationship between mRNA and protein levels, but that the three cell lines with the highest mRNA levels do not show further increased FAT protein levels (**Fig. 3**).

The presence of the FAT protein in the transfected cell lines was also studied by immunofluorescent labeling, using a monoclonal antibody against human CD36. Results

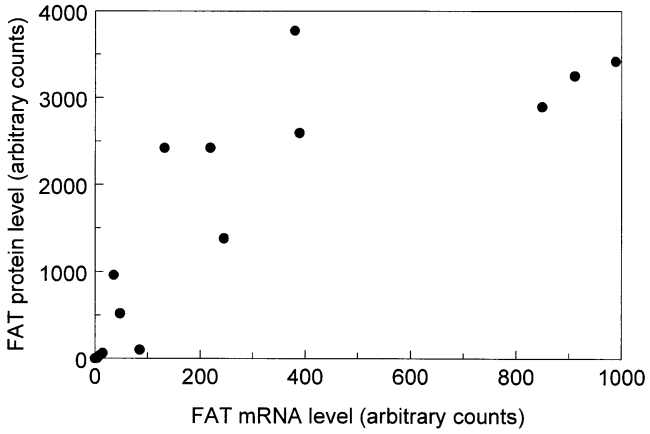


**Fig. 3.** Scatter diagram of the semi-quantitative protein level of FAT (determined densitometrically from Western blot) as function of the semi-quantitative mRNA level (determined using phosphorimager) in 15 FAT-transfected cell lines derived from H9c2.

of immunolabelling a positive (1A3) and a negative (3B3) cell line, as judged by both Northern and Western blot analyses, is shown in **Fig. 4**. A positive signal with the monoclonal antibody against FAT was found in 1A3 cells, while 3B3 cells were negative. In addition, FAT could be detected on the surface of living 1A3 cells (impermeable to antibodies), even after mild trypsinization (data not shown), which is in accordance with the known resistance to proteases of this particular protein (16, 17). Together, these experiments indicate that FAT is expressed as a plasma membrane protein, with the antibody binding site on the extracellular side of the transfected cells.

### Characterization of palmitate uptake and metabolism

The kinetics of palmitate uptake and the cellular fate of the palmitate taken up were studied in untransfected H9c2 cells and in two of the transfected cell lines, namely 1A3 (high FAT expression) and 3B3 (no FAT expression). Palmitate uptake was measured as a function of time, using a fixed palmitate:BSA ratio of 1:3 (0.1 mm palmitate and 0.3 mm BSA), which represents a physiologically relevant condition. The uptake of palmitate increased linearly in time in both untransfected H9c2 cells and transfected cell lines during at least the first 30 min, with the rate of uptake amounting to approximately 20 nmol/min per g of wet mass (data not shown).

Palmitate uptake rates then were studied at different palmitate concentrations using a constant BSA concentration, to reveal possible saturation kinetics. For practical reasons, with this set of experiments we used a BSA concentration of 0.12 mm, which still allows us to study the influence of the non-protein-bound palmitate concentration on the uptake rate because this latter palmitate concentration is a function of the palmitate:BSA molar ratio (25). Indeed, the palmitate uptake rates of the cell lines were similar when the total amount of palmitate and BSA was varied by a factor of 2.5 while keeping their ratio, and thus the non-protein-bound palmitate concentration, constant, that is at 0.04 mm palmitate/0.12 mm BSA (**Fig. 5**) and 0.1 mm palmitate/0.3 mm BSA (**Fig. 6**). As shown in the insert of Fig. 5, in all three cell lines uptake rates of palmitate were saturable with increasing non-protein-bound palmitate concentrations. The data were studied according to Zivin and Waud (24) using the Eadie-Hofstee

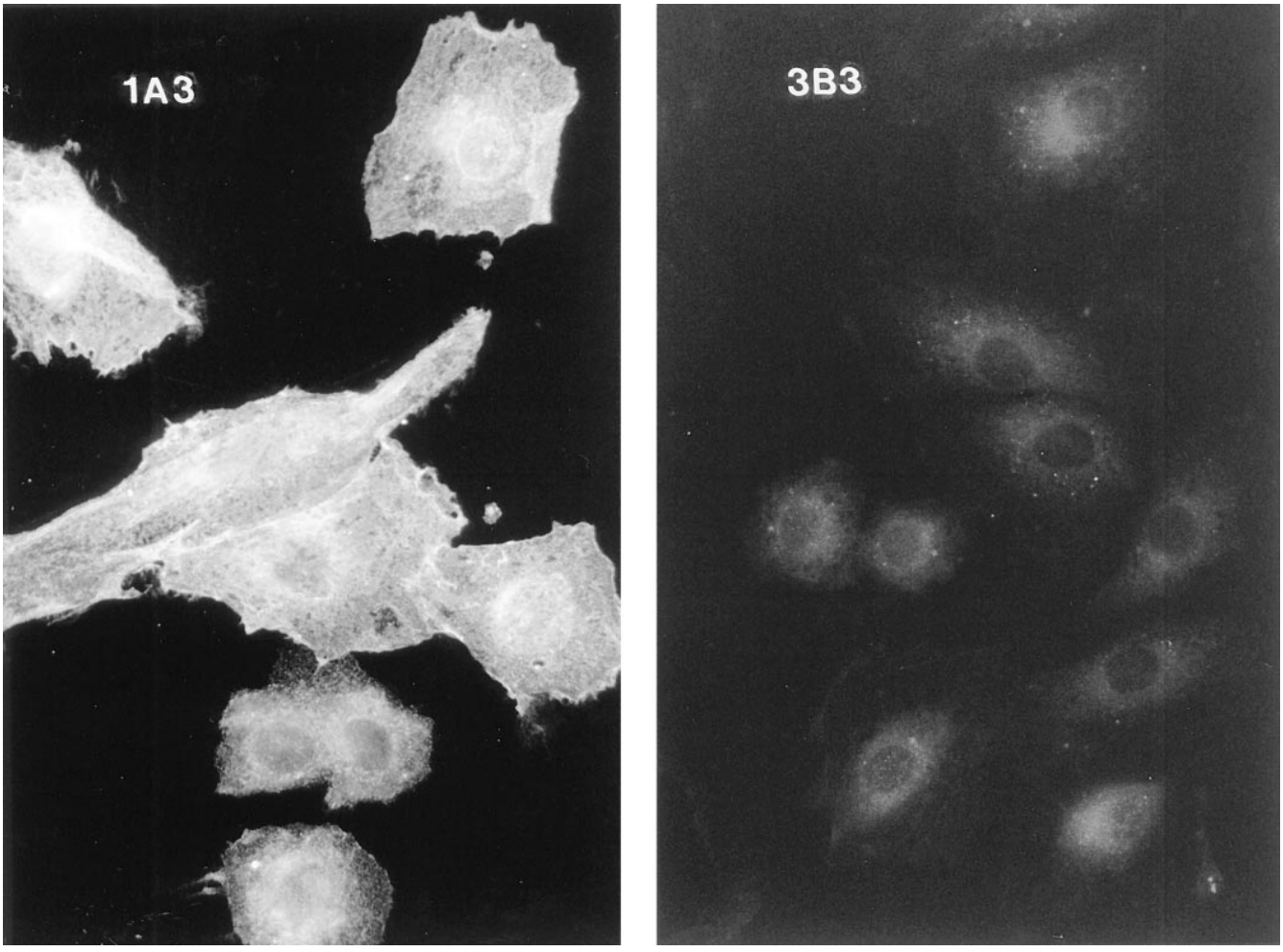


**Fig. 4.** Labeling of FAT in 1A3 and 3B3 cells by immunofluorescence using monoclonal 131.4 as primary antibody, and an FITC-labeled rabbit anti-mouse Ig as secondary antibody. Before incubation with the antibodies, cells were fixed with 1% paraformaldehyde.

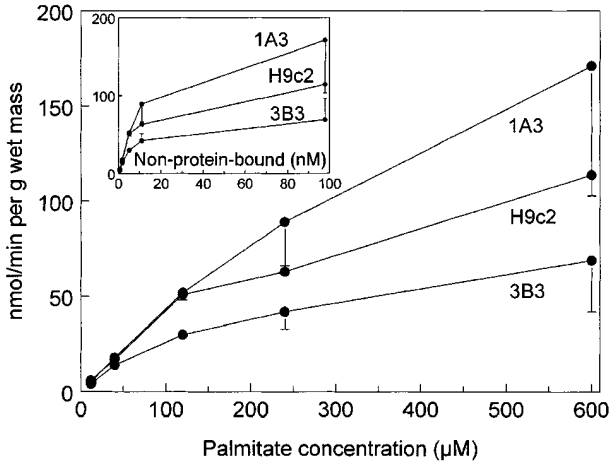


**Fig. 5.** Palmitate uptake rate of non-transfected H9c2, 1A3 (high FAT expression) and 3B3 (no FAT expression) as function of the palmitate concentration using a constant BSA concentration of 0.12 mm. Insert: palmitate uptake rate as function of the non-protein bound palmitate concentration, as calculated from the palmitate-BSA ratio according to Richieri, Anel, and Kleinfeld (25). The uptake rates were determined after 3- or 10-min incubation periods. Data are mean ± SD for n = 2 experiments except for H9c2, n = 1. All experiments were performed in duplicate.

plot to calculate $V_{max}$ and $K_m$ of the uptake process. $V_{max}$ of palmitate uptake was 130, 70, and 175 nmol/min per g wet mass in H9c2, 3B3, and 1A3 cells, respectively. The $K_m$ was approximately 11 nm in all cases.

The observed saturation of the palmitate uptake rates in H9c2 cells (as well as the transfected cells) at high palmitate concentrations can be caused by either protein-mediated uptake or by rate-limiting intracellular palmitate handling (cytoplasmic transport and metabolic conversion). As FAT is not expressed in H9c2 cells, we studied whether other candidate fatty acid transporters were expressed in these cells. For this, we performed Northern blot analyses with the cDNAs of fatty acid-transport protein (FATP) (26), and of plasmalemmal fatty acid-binding protein (FABPpm) which is reported to be identical to mitochondrial aspartate aminotransferase (mAAT) (27, 28). Both FATP and FABPpm were found to be expressed in H9c2 cells and also in all of the FAT-transfected cell lines (data not shown). Thus, we subsequently studied the possible involvement of FATP, FABPpm, or other proteins in palmitate uptake by H9c2 cells. For this, the cells were incubated with 0.4 mm of either phloretin (non-specific inhibitor of membrane processes), sulfo-*N*-succinimidyl-oleate (SSO, a reactive long-chain fatty acid derivative), or 4,4′-diisothiocyano-stilbene-2,2′-disulfonic acid (DIDS, an irreversible inhibitor of transport systems in many cell types). These three agents have previously been used with adipocytes and cardiac myocytes as inhibitors of protein-mediated long-chain fatty acid uptake (9, 12, 29, 30). Palmitate uptake rates in H9c2 cells were, however, not affected by either of these interventions. In addition, incubation of 1A3 and 3B3 cells with SSO did not result in significantly decreased palmitate uptake rates (data not shown). Uptake of 2-deoxy-d-glucose, which was measured as a control for the action of phloretin, was not affected by SSO nor by DIDS, but was inhibited by 55% when phloretin was added to the incubation medium (data not shown). Similar experiments, performed in isolated cardiac myocytes in an earlier study (9), resulted in a 80% and 50% decreased rate of palmitate uptake after incubation with phloretin or SSO, respectively. Our present results indicate that although the putative fatty acid transporters FABPpm and FATP are present, the bulk of palmitate uptake in H9c2 cells is not protein-mediated. This implies that the observed saturation of palmitate uptake rate at high palmitate concentrations is most likely caused by intracellular palmitate handling becoming rate limiting.

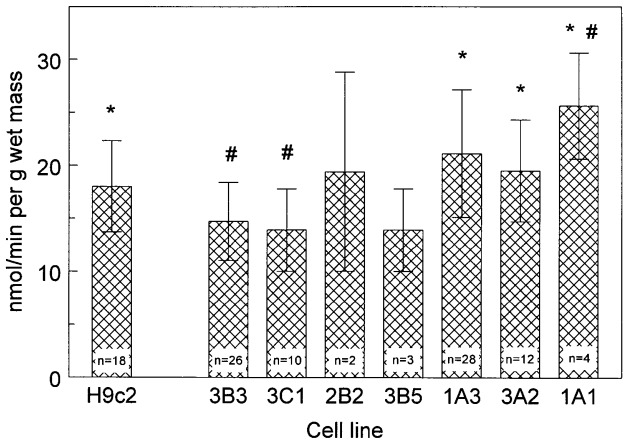


**Fig. 6.** Palmitate uptake rate of H9c2 cells and seven FAT-transfected cell lines. Cells were incubated with palmitate:BSA ratio of 1:3 (palmitate 0.1 mm and BSA 0.3 mm) and the uptake rate was determined after 3 or 10 min incubation. The studied FAT-transfected cell lines are listed from left to right in sequence of increasing FAT expression (as was determined with Northern and Western blotting, see also Figs. 2 and 3). 3B3 and 3C1 do not contain measurable amounts of FAT expression, neither on mRNA nor on the protein level. Data are mean ± SD for the indicated number of experiments. # Indicates statistically different from H9c2 and * indicates statistically different from 3B3 (no FAT expression) ($P < 0.05$).

Palmitate incorporation into lipid pools was studied in 1A3 and 3B3 cells at a palmitate:BSA ratio of 1:3 (0.1 mm and 0.3 mm, respectively) using both 3 and 10 min as incubation times. At both time intervals, and for both 1A3 and 3B3 cells, approximately 75% of the palmitate taken up was found to be incorporated into phospholipids, 20% was included in triacylglycerols, while the remaining 5% remained unesterified. No oxidation products could be detected in the cell lines, not even after 30 min incubation.

### Effect of FAT expression on palmitate uptake

The results of the kinetic studies were used to select conditions under which the intracellular palmitate handling capacity was not fully utilized. Therefore, palmitate uptake rates were measured using a ratio of palmitate:BSA of 1:3 (0.1 mm and 0.3 mm, respectively). At this ratio, the uptake rates of palmitate increased linear with the non-protein bound palmitate concentration and were approximately 20% of $V_{max}$ (Fig. 5). To test the hypothesis that

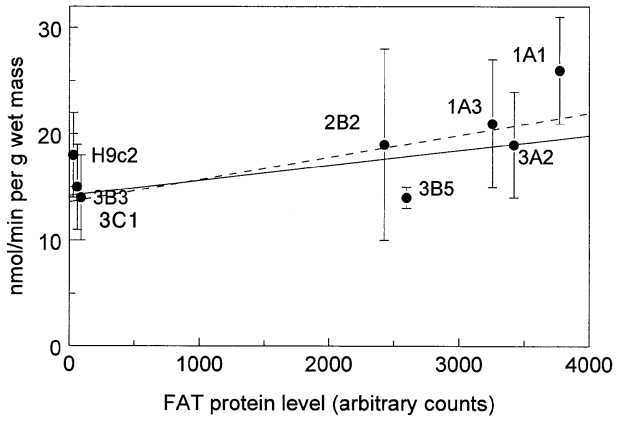


**Fig. 7.** Scatter diagram showing the relationship between FAT expression as measured using Western blot analysis and the palmitate uptake rate. No statistically significant correlation ($P > 0.05$) was found between FAT protein and palmitate uptake in the FAT-transfected cell lines, neither when the 1A1 cell line was included nor when it was excluded from the calculations (note that the untransfected H9c2 cell line was included in none of the calculations). Solid line is the calculated regression curve without 1A1: $y = 0.0014 \cdot x + 14.2$. Dashed line represents the calculated regression curve including 1A1 as data point: $y = 0.0021 \cdot x + 13.6$.

FAT expression increases the palmitate uptake rate of H9c2 cells, the uptake rate of palmitate was measured in a subset of the FAT-transfected cell lines. Figure 6 shows the palmitate uptake rates of H9c2 and of 7 of the transfected cell lines, listed in order of increasing FAT protein expression as judged by Western blot analysis (cf. Figs. 2 and 3). Palmitate uptake rates varied by approximately a factor of 2. Although some statistically significant differences in palmitate uptake rate among the cell lines were observed (Fig. 6), no statistically significant correlation was found between FAT expression and palmitate uptake rate, as shown in **Fig. 7**.

## DISCUSSION

The aim of the present study was to investigate the role of FAT in the uptake of long-chain fatty acids by muscle cells. FAT was previously identified in rat adipocytes (10, 12) and is probably a species homologue of the human leukocyte differentiation antigen CD36, a receptor protein present, among others, on monocytes, platelets, and endothelial cells (16, 17). FAT is also expressed in myocytes (11) where it may belong to a set of proteins that are putatively involved in the transfer of long-chain fatty acids across the plasma membrane (2). Two other candidate proteins which are expressed in myocytes are fatty acid-transport protein (FATP) (26) and the plasmalemmal fatty acid-binding protein (FABPpm) (8). According to some investigators the latter protein is identical to the mitochondrial isoform of aspartate aminotransferase (mAAT) (27, 31).

H9c2 cells were chosen as a model because they possess several characteristics of both cardiac and skeletal muscle cells, but have the advantage of being immortalized. In addition, untransfected H9c2 cells do not express FAT in measurable amounts as was found using Northern and Western blot analyses. Therefore, stable transfection of FAT in these cells would provide a model to study in more detail the function of FAT in long-chain fatty acid uptake by muscle cells. In the present study, we succeeded in stably transfecting H9c2 cells with FAT cDNA, resulting in 15 cell lines showing varying levels of FAT expression. Some of the cell lines (i.e., 3B3 and 3C1) showed no detectable FAT expression, while others gave strong signals on both Northern and Western blot analyses, the highest expression levels being of magnitude similar to that found in rat heart (see Figs. 1 and 2). FAT protein was expressed as a plasma membrane protein as was found by immunolabeling of (living) FAT-transfected cells with anti-FAT antibodies. Further proof of the plasma membrane localization and correct conformation of FAT in the transfected cell lines was obtained when we were able to select antibodies from a phage–antibody library using living 1A3 cells (M. M. A. L. Pelsers, H. R. Hoogenboom, and J. F. C. Glatz, unpublished observations). These antibodies were found to recognize both rat FAT and human CD36, indicating that the FAT expressed in the transfected cell lines was immunochemically similar to naturally occurring FAT. We cannot exclude, however, minor differences of the transfected FAT protein, e.g., with respect to glycosylation, which might affect its function.

We first measured the palmitate uptake characteristics in H9c2 cells and in the FAT-transfected cell lines. Palmitate uptake was linear in time for at least 30 min and the uptake rate was approximately 20 nmol/min per g of wet mass. This uptake rate is of magnitude similar to the initial uptake rate of palmitate observed in isolated quiescent cardiac myocytes, measured under comparable conditions (9).

The palmitate uptake rate was saturable with increasing non-protein-bound palmitate concentration in all the cell lines studied (Fig. 5). The saturability can either be caused by protein-mediated transport or be the result of rate-limiting intracellular fatty acid handling (dissociation from the inner leaflet of the plasma membrane into the cytoplasm, transcytoplasmic transport, metabolic conversion). Because we could establish the presence of both FATP and FABPpm in H9c2 cells and in FAT-transfected cell lines, we tested the contribution of these two proteins and possible other (unknown) proteins to the palmitate uptake by H9c2 cells. Incubating the cells with either phloretin, SSO, or DIDS did not affect the palmitate uptake rate. Therefore, we conclude that plasma membrane-associated proteins are not involved in the bulk of palmitate uptake by H9c2 cells, which by inference must occur mainly by passive diffusion, and that limitations of intracellular fatty acid handling probably are responsible for the observed saturability with high palmitate concentrations.

Using conditions under which the intracellular palmitate handling would not be rate limiting (0.1 mm palmitate complexed to 0.3 mm BSA, i.e., at a low but physiologically relevant non-protein-bound palmitate concentration) we measured the palmitate uptake rate in 7 out of the 15 FAT-

transfected cell lines. There was some variation in palmitate uptake rate among the cell lines and statistically significant differences were observed between individual cell lines. However, it should be kept in mind that differences between individual cell lines may be caused by transfection and/or selection rather than by FAT-expression. Thus, to study the effect of FAT-expression on palmitate uptake rate in the transfected cell lines, we examined whether a relationship would exist between the level of FAT protein expression and the palmitate uptake rate in a number of these FAT-transfected cell lines. The outcome was that no significant correlation could be found between FAT expression and palmitate uptake rate in the cell lines studied (Fig. 7).

In contrast, in a recent study, expression of CD36 in Ob17PY cells was observed to increase uptake of both palmitate and oleate (14). In this study, one of the three cell lines obtained after stable transfection of the Ob17PY cells with FAT showed considerably increased rates of uptake of palmitate and oleate, while in the other two cell lines only a marginal increase was observed (14). A possible explanation for the contradictory results of the present study and that of Ibrahimi et al. (14) might be that Ob17PY cells express a protein (or set of proteins) that serves as an obligatory partner for FAT in long-chain fatty acid uptake, while such protein is lacking in H9c2 cells. For muscle cells, a candidate protein could be the intracellular heart-type fatty acid binding protein (H-FABP) which is abundant in both heart and skeletal muscles (1, 11) but is not expressed in H9c2 cells (unpublished observations). Both a co-expression (11, 32) and association (32) between FAT and H-FABP has been reported, and this association was hypothesized to play a role in long-chain fatty acid uptake (1). In addition, in intestinal epithelium, FAT was found to be co-expressed with intestinal- and liver-type FABPs (33).

In conclusion, we found that H9c2 cells show a palmitate uptake rate of magnitude similar to that of cardiac myocytes, and that this uptake is saturable with increasing non-protein-bound palmitate concentrations, but most likely is not mediated by plasma membrane-associated proteins. Introducing high FAT-expression in these cells did not result in significantly increased palmitate uptake rates. The FAT-transfected cell lines will form a suitable model to study the role of possible obligatory partners for FAT in long-chain fatty acid uptake.

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