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FAT/CD36 is located on the outer mitochondrial membrane, upstream of long-chain acyl-CoA synthetase, and regulates palmitate oxidation

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FAT/CD36 (fatty acid translocase/Cluster of Differentiation 36), a plasma membrane fatty-acid transport protein, has been found on mitochondrial membranes; however, it remains unclear where FAT/CD36 resides on this organelle or its functional role within mitochondria. In the present study, we demonstrate, using several different approaches, that in skeletal muscle FAT/CD36 resides on the OMM (outer mitochondrial membrane). To determine the functional role of mitochondrial FAT/CD36 in this tissue, we determined oxygen consumption rates in permeabilized muscle fibres in WT (wild-type) and FAT/CD36-KO (knockout) mice using a variety of substrates. Despite comparable muscle mitochondrial content, as assessed by unaltered mtDNA (mitochondrial DNA), citrate synthase, β-hydroxyacyl-CoA dehydrogenase, cytochrome *c* oxidase complex IV and respiratory capacities [maximal OXPHOS (oxidative phosphorylation) respiration] in WT and KO mice, palmitate-supported respiration was 34 % lower in KO animals. In contrast, palmitoyl-CoA-supported respiration was unchanged. These results indicate that FAT/CD36 is key for palmitate-supported respiration. Therefore we propose a working model of mitochondrial fatty-acid transport, in which FAT/CD36 is positioned on the OMM, upstream of long-chain acyl-CoA synthetase, thereby contributing to the regulation of mitochondrial fatty-acid transport. We further support this model by providing evidence that FAT/CD36 is not located in mitochondrial contact sites, and therefore does not directly interact with carnitine palmitoyltransferase-I as original proposed.

Key words: carnitine palmitoyltransferase I, fatty acid translocase/Cluster of Differentiation 36 (FAT/CD36), mitochondrial fatty-acid transport, mitochondrial membrane, mitochondrial respiration.

INTRODUCTION

FAT/CD36 (fatty acid translocase/Cluster of Differentiation 36) is a class B scavenger receptor with strong homology with human CD36 (glycoprotein IV) and has been shown to function as a plasma membrane LCFA (long-chain fatty acid) transporter in various tissues, including skeletal muscle, heart, liver, adipose tissue and the small intestine [1–7]. Additionally, in heart and skeletal muscle, intracellular pools of FAT/CD36 exist which can be induced to translocate to the plasma membrane to regulate LCFA uptake through the activation of several signalling cascades, including AMPK (AMP-activated protein kinase) [8], CaMKK (Ca^{2+}/calmodulin-dependent protein kinase kinase) [9], ERK1/2 (extracellular-signal-regulated kinase 1/2) [10] and insulin [11].

FAT/CD36 has also been found on the mitochondrial membranes of heart, liver and skeletal muscle [12,13], where it co-immunoprecipitates with CPT-I (carnitine palmitoyltransferase I) [3,12,14] and has been hypothesized to influence mitochondrial fatty-acid transport and oxidation [3,14–19]. However, the functional role of FAT/CD36 in mitochondrial fatty-acid oxidation has remained controversial. For example, studies examining the function of mitochondria isolated from FAT/CD36-KO (knockout) mice have yielded conflicting results with reductions in palmitate oxidation reported in one study [18], and in contrast, unaltered P-CoA (palmitoyl-CoA; 20 μM) and palmitoyl carnitine (40 μM) state III respiration rates in another [20]. These divergent results may reflect the substrates selected, as it is currently unknown where FAT/CD36 resides on mitochondrial membranes, and selective substrates (including P-CoA and palmitoyl carnitine) may have bypassed the regulatory step exerted by FAT/CD36.

In addition to the equivocal reports examining mitochondrial function in FAT/CD36-KO mice, a recent investigation has also failed to detect FAT/CD36 on mitochondrial membranes utilizing both immunoblotting and fluorescence immunocytochemistry techniques [21]. Indeed, contamination of other cell fractions has been an issue when isolating skeletal muscle mitochondria. These recent data, in combination with the ill-defined functional role of FAT/CD36 on mitochondrial membranes, raises the possibility that FAT/CD36 does not reside on mitochondrial membranes, and previous reports may have observed contamination from regions within the cell that contain FAT/CD36, in particular, the plasma membrane.

Since there is controversy as to whether FAT/CD36 is (a) located on mitochondrial membranes and (b) whether this protein plays a functional role in contributing to the regulation of mitochondrial fatty-acid oxidation, we have isolated mitochondria, purified them, and further subfractionated the mitochondria to determine the location of FAT/CD36 within this organelle. In addition, we have determined respiration rates of mitochondria in the presence

Abbreviations used: ACS, acyl-CoA synthetase; Cav-3, caveolin 3; COXIV, cytochrome *c* oxidase complex IV; CPT-I, carnitine palmitoyltransferase I; CS, citrate synthase; ETC, electron transport chain; FABPc, fatty-acid-binding protein c; FAT/CD36, fatty acid translocase/Cluster of Differentiation 36; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; GLUT4, glucose transporter 4; β-HAD, β-hydroxyacyl-CoA dehydrogenase; IMM, inner mitochondrial membrane; KO, knockout; LCFA, long-chain fatty acid; MCT, monocarboxylate transporter; mtDNA, mitochondrial DNA; OMM, outer mitochondrial membrane; P-CoA, palmitoyl-CoA; PDHE1α, pyruvate dehydrogenase E1 α-subunit; RCR, respiratory control ratio; SERCA-2, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 2; SUIT, substrate-uncoupler-inhibitor titration; TEM, transmission electron microscopy; WT, wild-type.

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of various substrates (including palmitate) in permeabilized muscle fibres from WT (wild-type) and FAT/CD36-KO mice. These studies provide evidence that (i) FAT/CD36 is located on the OMM (outer mitochondrial membrane) upstream of long-chain ACS (acyl-CoA synthetase) and (ii) that FAT/CD36 is involved in regulating mitochondrial fatty-acid oxidation.

EXPERIMENTAL

Animals

Female rats (8–10 weeks of age and weighing ~175 g) were housed in a climate- and temperature-controlled room, on a 12-h/12-h light/dark cycle, with standard chow and water provided *ad libitum*. FAT/CD36-KO mice were obtained from Dr Maria Febbraio (Cleveland Clinic, Cleveland, OH, U.S.A.) [5]. Breeding of WT and KO mice was conducted on site at the University of Guelph. Age-matched (8–10 weeks of age) female WT (20.6 ± 0.8 g) and KO (21.2 ± 1.1 g) mice were used in the present study. This study was approved by the University of Guelph Animal Care Committee.

Isolation of highly purified mitochondria, cytosolic compartment, plasma membrane and homogenate from skeletal muscle

The plasma membrane and muscle homogenate samples were prepared as described previously [22,23]. Differential centrifugation was used to obtain intermyofibrillar mitochondrial fractions, and all procedures were identical with those that we have previously published [18]. The cytosolic fraction was obtained by generating plasma membrane vesicles followed by three freeze–thaw cycles (to fractionate the plasma membrane from the cytosol) and then high-speed centrifugation to separate out the respective fractions.

OMM digestion

To determine the subcellular location of FAT/CD36 on mitochondrial membranes, the OMM of mitochondria was selectively digested by incubating with, or without, digitonin. Mitochondria were exposed to various concentrations of digitonin for 15 min (0, 25, 50, 100, 200 and 400 μg/mg of mitochondria), and thereafter centrifuged at 10 000 ***g***, resuspended and markers of IMM (inner mitochondrial membrane) and OMM via Western blot analysis (described below) were quantified. We compared the dose–response results as the ratio of FAT/CD36/PDHE1α (pyruvate dehydrogenase E1α subunit) to ensure that the digitonin treatment was selectively removing the OMM and not degrading the IMM. The FAT/CD36/PDHE1α ratio for the respective digitonin concentrations used were as follows: 1.00, 0.82, 0.64, 0.54, 0.45 and 0.35. These results indicated that as we increased the concentration of digitonin we concentrated the IMM proteins within the sample, and therefore the ratio between FAT/CD36 and PDHE1α decreased, as expected. The digitonin concentration of 100 μg/mg of mitochondria was used for subsequent experiments and is identical with that previously used elsewhere [24]. In addition, we determined whether proteinase K exposure altered either OMM or IMM proteins. For these studies, mitochondria were incubated for 15 min at various concentrations of proteinase K (ranging from 3.125 ng/ml up to 50 ng/ml), followed by the addition of 2 mM PMSF, as described previously [25]. In contrast with digitonin, across all concentrations examined, proteinase K did not alter either FAT/CD36 or PDHE1α contents, and therefore the ratio of FAT/CD36/PDH was constant. We therefore did not perform subsequent experiments in the presence of proteinase K.

OMM and contact site isolation

Following isolation of mitochondria (as described above), separation of OMM and contact sites was achieved via a swell/shrink method, followed by further differential centrifugation [26]. Separation of OMM and contact sites was performed via discontinuous sucrose-gradient centrifugation. The sucrose gradient was 1.2 ml of 51.3 %, 37.7 % and 25.2 % sucrose in 20 mM potassium phosphate. Addition of 1 ml of the crude membrane fraction was loaded and centrifuged for 60 min at 4 °C and 36 000 rev./min. The membranous material at the interface between the 25.2 %/37.7 % (purified OMM) and the 37.7 %/51.3 % (contact-site fraction) phases were collected and diluted by adding 10 ml of 20 mM potassium phosphate. The diluted fractions were then centrifuged for 1 h at 38 600 rev./min and resuspended in 100 μl of 20 mM potassium phosphate for subsequent analysis. To obtain sufficient mitochondria, skeletal muscle from ten animals was pooled for each independent experiment.

Co-immunoprecipitation

Co-immunoprecipitation experiments were performed using the Pierce Classic IP Kit (Thermo Scientific) according to the manufacturer's instructions. Briefly, 200 μg of intact or fractionated mitochondrial protein was incubated with 4 μg of FAT/CD36 antibody (Santa Cruz Biotechnology) overnight, followed by a 1 h incubation at 4 °C in the presence of Protein L agarose beads (Santa Cruz Biotechnology). Following centrifugation at 1 000 ***g*** for 1 min, the eluant was kept as a control for subsequent Western blot analyses. The immune complex was then recovered by the addition of SDS loading buffer and boiling for 5 min.

Mitochondrial enzymatic activities

Muscle samples (~10 mg) were homogenized in 100 (w/v) of a 100 mM potassium phosphate buffer and used for the measurements of CS (citrate synthase) and β-HAD (β-hydroxyacyl-CoA dehydrogenase) [27,28]. Total CS activity was assayed spectrophotometrically at 37 °C at 412 nm, and β-HAD activity was measured at 340 nm (37 °C).

TEM (transmission electron microscopy)

TEM of muscle was performed as we have reported previously [22]. Briefly, samples were rapidly immersed in a fixing buffer, incubated overnight, and thereafter sections (100 nm) were cut and laid on to 200 mesh formvar/carbon copper grids and then stained with 2 % uranyl acetate and Reynold's lead citrate. Samples were viewed on a Philips CM 10 TEM at 80 kV, and images were obtained with an Olympus/SIS Morada CCD (charge-coupled device) camera using the Olympus/SIS iTEM software.

mtDNA (mitochondrial DNA)

mtDNA copy number was determined using real-time PCR, as described previously [22] using the following primers: NADH

dehydrogenase subunit 5 forward, 5′-GCAGCCACAGGAAAA-TCCG-3′, and reverse, 5′-GTAGGGCAGAGACGGGAGTTG-3′; and the solute carrier family 16 member 1 forward, 5′-TAG-CTGGATCCCTGATGCGA-3′, and reverse, 5′-GCATCAGAC-TTCCCAGCTTCC-3′.

Western blot analysis

Mitochondria and mitochondrial membrane protein samples were separated using SDS/PAGE as described previously [18]. The monoclonal antibody MO25 [29] was used to detect FAT/CD36. Commercially available antibodies were used to detect Bcl-2 (Santa Cruz Biotechnology), COXIV (cytochrome *c* oxidase complex IV; Invitrogen), complexes I, II, IV and V of the ETC (electron transport chain) (Mitosciences), CPT-I (Alpha Diagnostic), transferrin (Chemicon), calnexin (Sigma), GLUT4 (glucose transporter 4; Chemicon), Cav-3 (caveolin 3; BD Biosciences), PDHE1α (E1 α-subunit of PDH; Invitrogen), β-HAD (Abcam) and SERCA-2 (sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 2; Sigma). MCT (monocarboxylate transporter) 1 and 4 antibodies were gifts from Dr Hideo Hatta (University of Tokyo, Tokyo, Japan). Signals were detected using enhanced chemiluminesence (PerkinElmer Life Science) and were subsequently quantified by densitometry by Gene Tool according to the manufacturer's instructions (SynGene, ChemiGenius2, PerkinElmer).

Preparation of permeabilized fibres

The preparation of saponin-permeabilized fibres was based on previously published methods [30]. Following dissection, red gastrocnemius fibre bundles (~2 mg) were separated with fine forceps under a binocular dissecting microscope in BIOPS buffer (2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 5.77 mM Na_2ATP, 6.56 mM $MgCl_2 \cdot 6H_2O$, 20 mM taurine, 15 mM sodium phosphocreatine, 20 mM imidazole, 0.5 mM dithiothreitol and 50 mM Mes, pH 7.1). Following separation, fibre bundles were placed in BIOPS buffer containing 50 μg/ml saponin, agitated for 30 min and then washed in respiration buffer (MIRO5; 0.5 mM EGTA, 3 mM $MgCl_2 \cdot 6H_2O$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM Hepes, 110 mM sucrose and 1 g/l fatty-acid-free BSA, pH 7.1). Fibres were left in ice-cold MIRO5 until respiration analysis.

Mitochondrial respiration

Mitochondrial respiration was measured in permeabilized fibres from WT and FAT/CD36-KO mice by high-resolution respirometry (Oroboros Oxygraph-2 k) at 30 °C and room air saturation. Separate fibres from the same animal were used to determine (in duplicate): (i) palmitate-supported respiration, (ii) P-CoA-supported respiration or (iii) ETC function using a modified SUIT (substrate-uncoupler-inhibitor titration) protocol as recommended by Oroboros. To measure palmitate-supported respiration, MIRO5 + 1 mM ATP + 5 mM ADP, 2 mM malate, and 2 mM L-carnitine + 1 mM CoA was used as the respiration medium. Palmitate was then titrated automatically (Tip2K) every 5 min to generate a kinetic curve. To measure P-CoA-supported respiration, MIRO5 + 1 mM ATP + 5 mM ADP, 2 mM malate and 2 mM L-carnitine was used as the respiration medium. Pilot experiments titrating P-CoA (10 μM) every 5 min determined that 70 μM was optimal and was the concentration used for subsequent analysis. To measure complex I- and II-supported respirations, a modified SUIT method was used. MIRO5 was used as the respiration medium. Malate (2 mM) + glutamate (10 mM) were added to the chamber and complex I-supported respiration was determined following the addition of 5 mM ADP. Succinate (10 mM) was then added to determine complex I- and II-supported respiration. Subsequent titration of 1 μM FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) was then performed to estimate maximal ETC activity. Antimycin A (2.5 μM) was added to inhibit complex III for measurement of residual oxygen consumption. Cytochrome *c* was added following all experiments (palmitate, P-CoA and SUIT) to ensure OMM integrity.

RESULTS

Presence of FAT/CD36 on mitochondria in resting skeletal muscle

A recent report has suggested that the presence of FAT/CD36 on mitochondrial membranes is due to contamination [21]. Given the current controversy regarding the presence of FAT/CD36 on mitochondrial membranes, we first compared selected marker proteins of different subcellular compartments with our highly purified mitochondria (Figure 1, left-hand panel). Comparison of plasma membrane and highly purified mitochondrial fractions revealed the expected subcellular localization of sarcolemmal proteins to the plasma membrane and mitochondrial proteins to the isolated mitochondrial samples, validating our fractionation procedures (Figure 1, left-hand panel). Specifically, in highly purified mitochondrial samples, the absence of Cav-3, MCT1 and 4, and GLUT4 suggest a lack of sarcolemmal contamination, whereas the absence of SERCA-2, transferrin and calnexin suggest a lack of sarcoplasmic and endoplasmic reticulum contaminations respectively (Figure 1, right-hand panel). In contrast, the mitochondrial proteins PDHE1α and subunits of complex II and IV of the ETCs, as well as ATP synthase subunit α, were all highly expressed and enriched in our isolated mitochondria, despite using a much lower protein concentration (homogenate, 40 μg; mitochondria, 7.5 μg). Collectively, these data suggest that our isolation yielded highly purified mitochondria devoid of contamination by other subcellular components. FAT/CD36 was highly expressed in these mitochondrial samples, indicating that FAT/CD36 does reside on mitochondrial membranes in skeletal muscle.

Location of FAT/CD36 on mitochondrial membranes

Since FAT/CD36 was present in the mitochondrial fraction, we investigated the location of FAT/CD36 on rat mitochondrial membranes via two separate experiments. Following OMM-specific digestion by digitonin, FAT/CD36 and Bcl-2 (a protein located on the OMM [31]) were both reduced (~20 %, $P < 0.05$) (Figures 2A and 2B). In contrast, PDHE1α (an IMM-specific protein) and β-HAD (a mitochondrial matrix protein) were increased (~20 and 30 % respectively) following digitonin digestion (Figures 2C and 2D). To ensure constant loading, FAT/CD36 and PDHE1α were run on the same gel and the PVDF membrane was subsequently cut, enabling the detection of two proteins from one gel. In addition, Ponceau staining revealed equal loading of sample, allowing us to conclude that protein loading is not a potential confounding error. Collectively, these results indicate that the digitonin digestion was successful in concentrating the IMM by selectively removing a portion of the OMM, therefore suggesting that FAT/CD36 is located on the OMM.

The above conclusion was also confirmed following an extensive subfractionation procedure to isolate the OMM (see the Experimental section). Specifically, FAT/CD36 and Bcl-2 were both enriched in the OMM fraction (~50 %, $P < 0.05$) (Figure 3). In contrast, IMM proteins, subunits of complexes

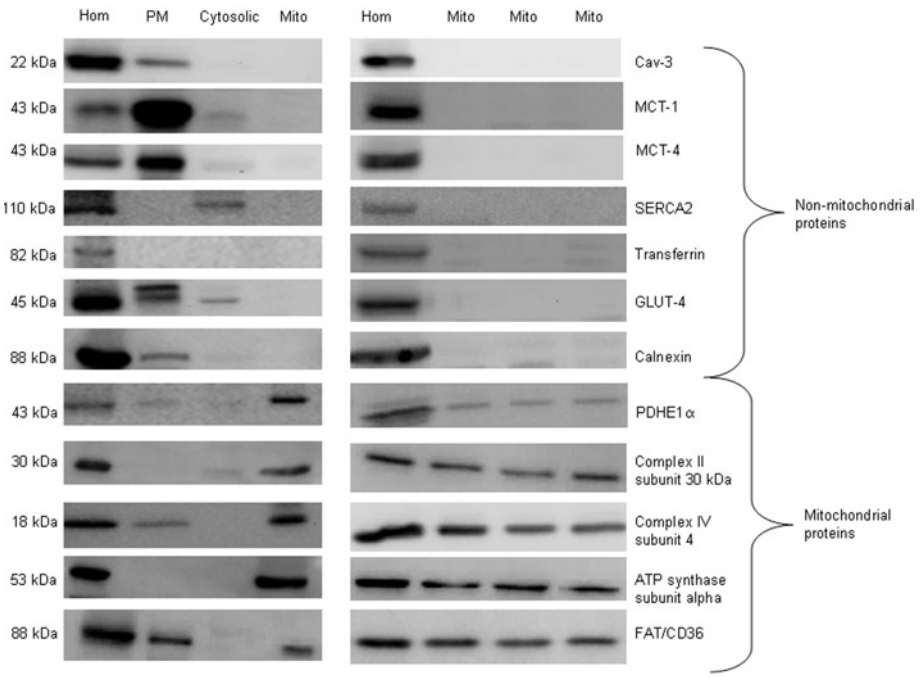

Figure 1 Characterization of isolated mitochondria

Mitochondria were isolated from Sprague–Dawley rats and were characterized using Western blot analysis. A total of 7.5 μg of mitochondrial protein (Mito) was loaded, whereas 40 μg of muscle homogenate (Hom), 10 μg of plasma membrane (PM) and 10 μg of the cytosolic fraction was loaded for comparison. The enrichment of mitochondrial proteins (PDHE1α, complexes II and IV, ATP synthase) and the absence of non-mitochondrial proteins (Cav-3, MCT-1, MCT-4, SERCA-2, transferrin, GLUT4 and calnexin) suggest that FAT/CD36 resides on mitochondrial membranes. The molecular mass in kDa is indicated on the left-hand side.

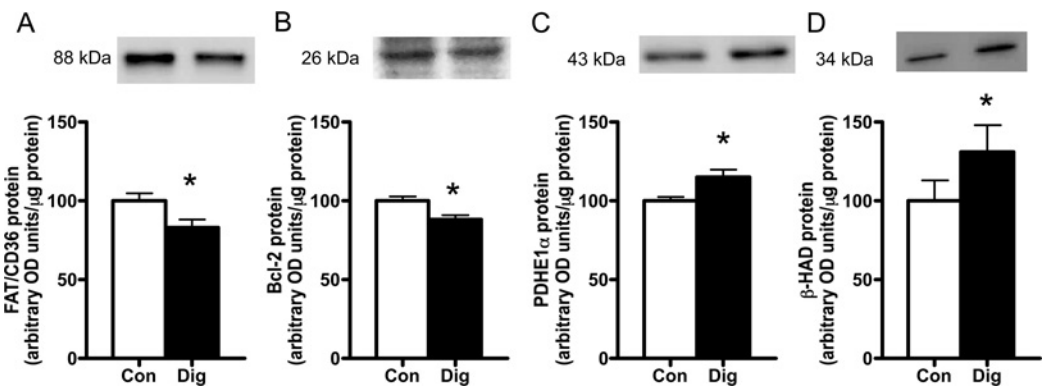

Figure 2 Effect of OMM digestion on FAT/CD36, Bcl-2, PDHE1α and β-HAD content

Equal protein amounts (7.5 μg) of (**A**) FAT/CD36, (**B**) Bcl-2, (**C**) PDHE1α and (**D**) β-HAD were loaded. Bcl-2 is known to reside on the OMM, whereas PDHE1α is known to reside on the IMM and β-HAD within the mitochondrial matrix. FAT/CD36 and Bcl-2 were significantly lower, whereas PDHE1α and β-HAD were significantly higher following digitonin (Dig) treatment. A Ponceau stain of the membrane was performed as a loading control. Taken together, these results suggest that FAT/CD36 resides on the OMM. $n = 4$ for all independent experiments, and values are means $\pm$ S.E.M., $^*P < 0.05$, control (Con) compared with digitonin-treated. The molecular mass in kDa is indicated.

I, II, III and IV, were all reduced ($\sim$75 %, $P < 0.05$) in the OMM fraction, suggesting that the OMM isolation protocol was successful (Figure 3). These two approaches, (digitonin digestion and extensive mitochondrial subfractionation), both indicate that FAT/CD36 resides on mitochondrial membranes, and more specifically, the OMM.

Palmitate respiration, mitochondrial content and mitochondrial function in FAT/CD36-KO mice

Given that FAT/CD36 was found on the OMM, we attempted to discern a potential function for this protein in regulating mitochondrial fatty-acid oxidation by examining respiration rates in permeabilized skeletal muscle fibres from WT and FAT/CD36-KO mice. The typical substrate used to study fatty-acid-supported bioenergetics is palmitoyl carnitine. However, palmitoyl carnitine bypasses all known potential regulation by proteins located on the OMM, and therefore, although this substrate is ideal within the context of studying ETC function, it is not appropriate for examining the regulation of mitochondrial LCFA transport. Therefore, in WT and FAT/CD36-KO mice, we measured palmitate-supported respiration. This approach revealed that maximal palmitate-supported respiration was 34 % lower ($P < 0.05$) in FAT/CD36-KO mice in the presence of a variety of concentrations of palmitate (between 75 μM and

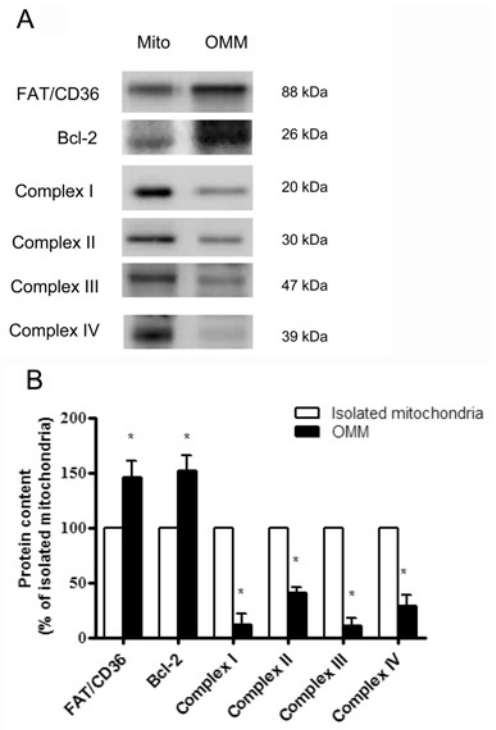

Figure 3 Expression of OMM and IMM proteins following OMM isolation

Equal amounts of protein (7.5 μg) were loaded and isolated mitochondria (Mito) were used as a standard. (**A**) Characteristic blots of FAT/CD36, Bcl-2 and complex I, II, III and IV subunits following OMM isolation. (**B**) Quantification of Western blots illustrated that FAT/CD36 and Bcl-2 were enriched, and complex I, II, III and IV subunits were reduced in the OMM fraction. $n = 3$ for all independent experiments, and values are means $\pm$ S.E.M., $^*P < 0.05$, isolated mitochondria compared with isolated OMM. The molecular mass in kDa is indicated.

525 μM; Figure 4). These results suggest that FAT/CD36 has a direct regulatory role in mitochondrial fatty-acid oxidation.

A decrease in mitochondrial content, independent of FAT/CD36, can also result in decreased rates of fatty-acid oxidation [32]. Therefore, to verify that FAT/CD36-KO mice do not have lower mitochondrial content, we determined markers of mitochondrial content and mtDNA in WT and KO mice. Analyses of CS, β-HAD, COXIV and mtDNA demonstrated that mitochondrial content was not different between WT and KO mice (Figure 5A). Furthermore we show that the morphology of subsarcolemmal and intermyofibrillar mitochondria are not different in WT and KO mice (Figure 5B). To confirm that reductions in palmitate-supported respiration in FAT/CD36-KO mice were not a result of mitochondrial dysfunction in FAT/CD36-KO mice, we determined respiration rates induced by a variety of substrates. WT and FAT/CD36-KO animals displayed similar rates of complex I (glutamate + malate)- and II (glutamate + succinate + malate)-supported respiration (Figure 6A). Maximal ETC activity as measured by the uncoupler FCCP (further confirming equivalent mitochondrial content), and basal respiration as estimated by the inhibition of complex III via antimycin A (Figure 6A) also demonstrated that mitochondrial content and function did not differ between WT and KO mice. In addition, the RCRs (respiratory control ratios) were not different between WT and FAT/CD36-KO mice (RCR = 8.0 ± 0.7 and 8.3 ± 0.6 respectively). Collectively, these data suggest that FAT/CD36 has a direct role in regulating mitochondrial fatty-acid oxidation.

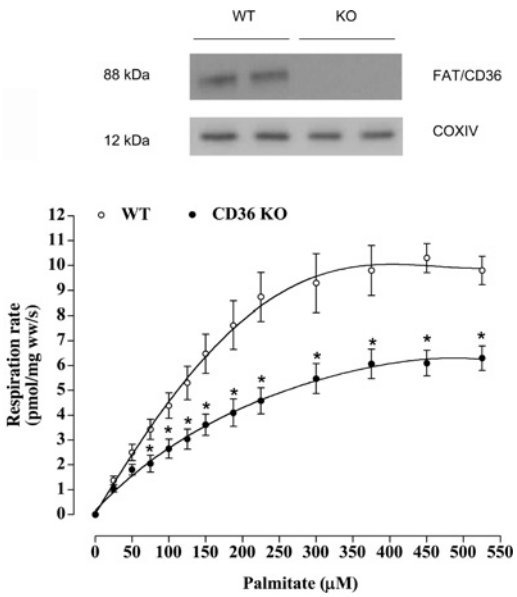

Figure 4 Palmitate-supported respiration in WT and FAT/CD36-KO mice

Palmitate-supported respiration was determined in the presence of malate (2 mM) and ADP (5 mM). Palmitate respiration was lower in the FAT/CD36-KO mice at all palmitate concentrations $\geqslant 75$ μM. $n = 6$–8 for all independent experiments, and values are means $\pm$ S.E.M., $^*P < 0.05$, WT compared with FAT/CD36-KO. Representative blots for FAT/CD36 and COXIV confirmed genotypes. The molecular mass in kDa is indicated.

P-CoA respiration in FAT/CD36-KO mice

The experiments above provide evidence that FAT/CD36 resides on the OMM and regulates palmitate-supported respiration. However, where along the OMM FAT/CD36 exerts this role is unclear. Therefore to determine whether FAT/CD36 is located proximal or distal to long-chain ACS (depicted in summary Figure 8), we next examined P-CoA-supported respiration in WT and FAT/CD36-KO mice. In contrast with palmitate-supported respiration, P-CoA respiration rates were not different in WT and FAT/CD36-KO animals (Figure 6B). The markedly divergent result in FAT/CD36-KO animals with respect to palmitate and P-CoA respiration rates strongly implies that FAT/CD36 influences fatty-acid oxidation proximal to ACS.

Contact sites and immunoprecipitation

Contact sites of mitochondrial membranes are the fusion points of the OMM and the IMM. It is known that CPT-I is prevalent in contact sites [33,34], and as such, it has been suggested that contact sites are important for facilitating fatty-acid transport into the mitochondria for subsequent oxidation [35]. Considering that FAT/CD36 has been observed to co-immunoprecipitate with CPT-I [3,12,14,19] it was somewhat unexpected that FAT/CD36 did not influence P-CoA respiration (substrate for CPT-I). Therefore we performed additional subfractionation experiments in rat skeletal muscle to isolate both the OMM and the contact-site fractions. These additional experiments revealed that CPT-I is located within contact sites, but not the OMM fraction (Figure 7A). In contrast, FAT/CD36 and Bcl-2 (which has previously been shown to be located on the OMM [31]) were enriched in the OMM, whereas their contents were reduced in the contact-site fraction (Figure 7A). To further supplement these findings, we performed co-immunoprecipitation experiments in intact and fractionated mitochondria. While FAT/CD36 and CPT-I co-immunoprecipitated in intact mitochondria (Figure 7B),

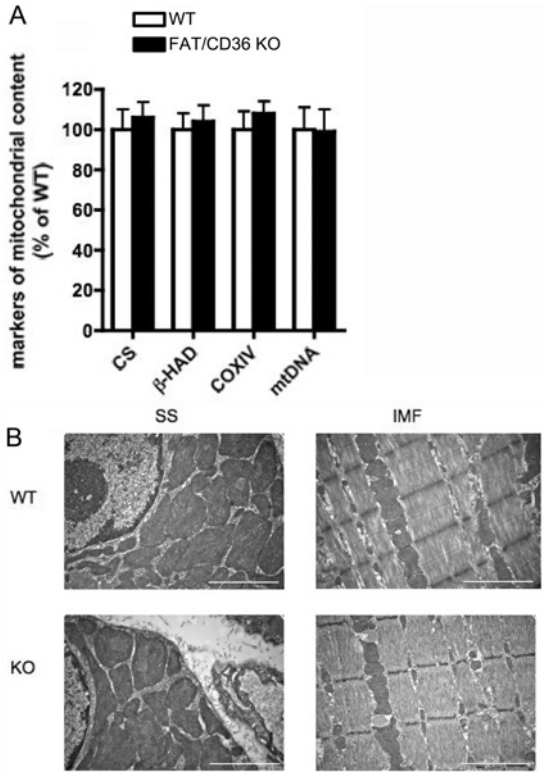

Figure 5 Mitochondrial morphology and content in WT and FAT/CD36-KO mice

Markers of whole-muscle mitochondrial content (**A**), including CS (absolute values; 80 ± 8 compared with 85 ± 6 mmol/kg of wet weight per min), β-HAD (absolute values; 25 ± 2 compared with 26 ± 2 mmol/kg of wet weight per min), COXIV and mtDNA, indicate that mitochondrial content is not altered in FAT/CD36-KO mice. In addition, TEM images of muscle taken at 25 000× magnification suggest the morphology of subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria are not different between WT and FAT/CD36-KO mice (**B**). Scale bar = 2 μm.

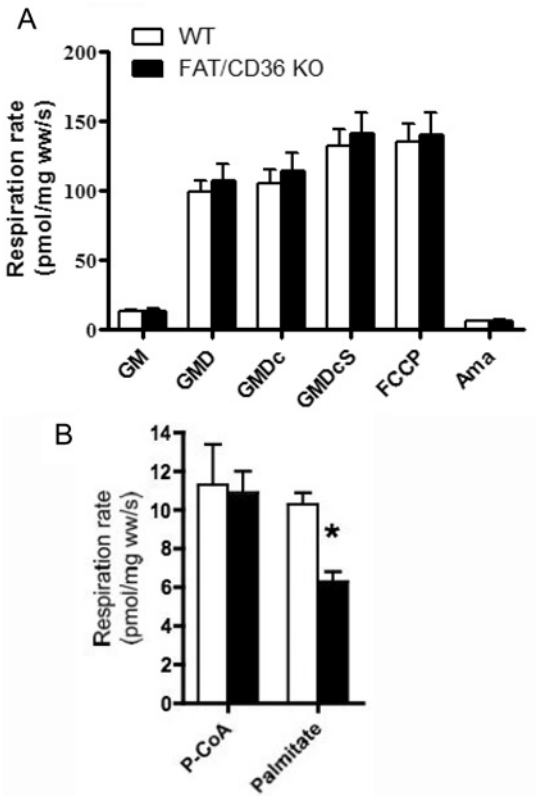

Figure 6 Mitochondrial respiration in WT and FAT/CD36-KO mice

(**A**) Conditions: malate (2 mM) + glutamate (10 mM), malate (2 mM) + glutamate (10 mM) + ADP (5 mM), malate (2 mM) + glutamate (10 mM) + ADP (5 mM) + succinate (10 mM), FCCP titrations and antimycin A (2.5 μM) were not different between strains. (**B**) P-CoA respiration rates were not different, whereas palmitate respiration rates were different between WT and FAT/CD36-KO mice. $n = 6$–8 for all independent experiments, and values are means $\pm$ S.E.M., $^*P < 0.05$, WT compared with FAT/CD36-KO.

these proteins did not co-immunoprecipitate in fractionated mitochondria (Figure 7C), suggesting that these proteins do not reside in the same region of the OMM. The observation that Bcl-2 co-immunprecipitates with FAT/CD36 (Figure 7C) indicates that regions of the OMM remained connected to FAT/CD36, further confirming that FAT/CD36 resides on the OMM.

DISCUSSION

In the present study, we investigated the presence of FAT/CD36 on skeletal muscle mitochondrial membranes and the functional role of this protein in mitochondrial fatty-acid oxidation. We provide evidence that FAT/CD36 (i) resides on mitochondrial membranes, specifically the OMM, and at this location (ii) influences mitochondrial fatty-acid oxidation proximal to ACS. Collectively, these two major findings establish that FAT/CD36 contributes to the regulation of mitochondrial fatty-acid oxidation.

FAT/CD36 on mitochondria

Our mitochondrial purification technique and contamination checks, in combination with the data from our previous work [12,18,36] and from four independent laboratories [3,14,19,20], indicate that FAT/CD36 is located on the mitochondrial membranes in several tissues, including skeletal muscle [3,12,15,18–20,32,36–38], liver [13] and heart [12] in a variety of species (rat, mouse and humans). It is unclear why one group [21] has been unable to replicate these findings. Nevertheless, there is now considerable evidence to support the presence of FAT/CD36 on mitochondria. This is further supported by our experimental approaches which suggest that FAT/CD36 is localized to the OMM in skeletal muscle. Moreover, previously in liver tissue, FAT/CD36 was identified on the OMM via MS [13]. The congruence in these reports provides evidence that FAT/CD36 is present on OMM.

Functional role of FAT/CD36

When comparing fatty-acid-stimulated respiration rates in FAT/CD36-KO with WT mice, we observed (i) lower palmitate-supported respiration rates in the KO mice and (ii) no difference in P-CoA-supported respiration rates between WT and KO mice. Our novel comparison between these two substrates (palmitate compared with P-CoA), combined with the use of an *in situ* model (permeabilized fibres), highlights the importance of selecting a substrate which is subject to the native regulation of a physiological system. Our observations are in agreement with two previous reports comparing isolated mitochondrial fatty-acid oxidation rates between WT and FAT/CD36-KO mice [18,20]. Holloway et al. [18] observed that basal levels of

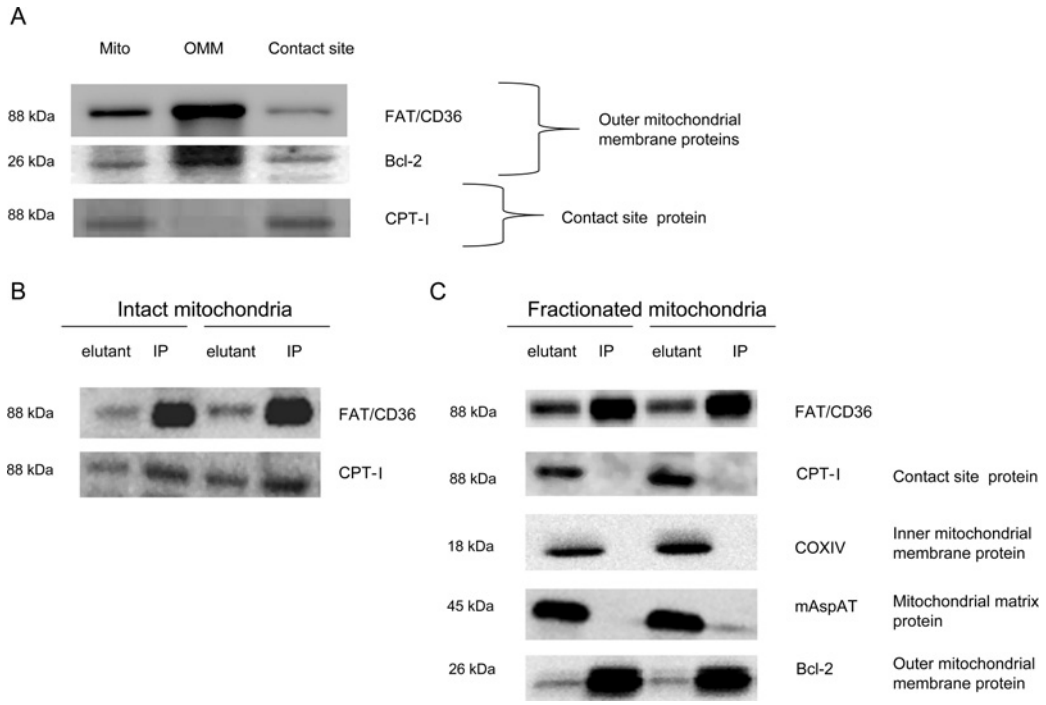

Figure 7 Distribution of FAT/CD36, Bcl-2 and CPT-I on mitochondrial membranes

(**A**) Equal protein amounts (7.5 μg) of isolated mitochondria (Mito), OMM and contact-site fractions were loaded. FAT/CD36 and Bcl-2 were enriched in the OMM fraction and reduced in the contact-site fraction. In contrast, CPT-I was enriched in the contact-site fraction and reduced in the OMM fraction. (**B**) Within intact mitochondria, FAT/CD36 and CPT-I co-imunoprecipitate together. (**C**) When mitochondria were fractionated, FAT/CD36 and CPT-I failed to co-immunoprecipitate. These results indicate that FAT/CD36 and CPT-I do not reside in the same region of the mitochondrial membranes. The molecular mass in kDa is indicated. IP, immunoprecipitate; mAspAT, mitochondrial aspartate aminotransferase.

mitochondrial palmitate oxidation rates were lower in KO mice, and King et al. [20] observed that P-CoA state III respiration rates were not significantly different between strains. Indeed, FAT/CD36 does appear to have a regulatory role in mitochondrial fatty-acid oxidation, as it is now apparent that ablation of FAT/CD36 reduces palmitate-supported respiration, but not P-CoA-supported respiration. As a result, we conclude that FAT/CD36 influences mitochondrial fatty-acid oxidation upstream of ACS.

CPT-I and FAT/CD36

CPT-I has long been viewed as the sole regulator of mitochondrial fatty-acid oxidation, owing to the integral role of this protein in LCFA movement into the mitochondrial matrix [39–42]. However, several studies have suggested that mitochondrial fatty-acid oxidation can be altered independent of CPT-I activity [15,18,43], and the results of the present study support this idea as palmitate oxidation was reduced in FAT/CD36-KO mice, a model known to have unaltered CPT-I activity [18]. Other mechanisms can/may also influence mitochondrial fatty-acid oxidation, including putative fatty-acid transport proteins such as the FATP family [19,44], the acetylation status of mitochondrial proteins [45,46], complex I glutathionylation [47] and/or phosphorylation of ETC complexes [48]. Clearly more work on the complexities surrounding the role of 'non-CPT-I' proteins and processes is necessary to unravel the complete regulation of mitochondrial fatty-acid metabolism, although evidence is mounting to suggest that FAT/CD36 contributes at this level.

Originally CPT-I and FAT/CD36 were hypothesized to interact directly due to positive co-immunoprecipitation results from a number of laboratories [3,12,14]. However, the results of the present study do not support the belief that CPT-I and FAT/CD36 directly interact, as CPT-I, but not FAT/CD36, was found within contact sites. Furthermore, when the mitochondrial membranes were sufficiently fractionated, FAT/CD36 and CPT-I did not co-immunoprecipitate, suggesting that these proteins are not in close proximity and do not directly interact. Consequently, we interpret the previous co-immunoprecipitation data, in addition with our own, as providing further support that FAT/CD36 is located on the OMM, rather than directly interacting with CPT-I within the contact sites.

Proposed working model

Proteins involved in the movement of fatty acids across cell membranes (such as FAT/CD36) have been hypothesized to act as LCFA acceptors to promote binding to downstream proteins as opposed to typical transport proteins which create a pore in the membrane (i.e. GLUT4 [38,49]). At the sarcolemma, FAT/CD36 has a large hairpin loop projecting into the interstitial space, which may function to accept LCFA from albumin and facilitate LCFA delivery and insertion into the outer leaflet of the plasma membrane. This process has been proposed to increase rates of 'flip-flop' across the membrane and ultimately rates of fatty-acid transport [50,51]. At the mitochondria, FAT/CD36 may function in a similar fashion, and we hypothesize a working model for fatty acid entry into the mitochondria (Figure 8). Specifically, we propose that FAT/CD36 accepts LCFA from FABPc

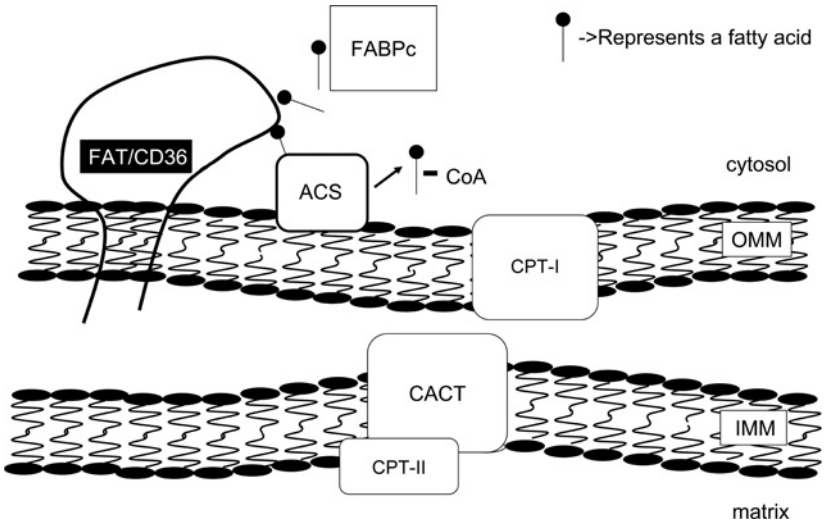

Figure 8 Proposed mechanism of action regarding FAT/CD36 involvement in LCFA entry into the mitochondria

In the present study we have found that FAT/CD36 is localized on the OMM and that ablating FAT/CD36 reduces palmitate-, but not P-CoA-, supported respiration. Therefore we propose that FAT/CD36 is located upstream of long-chain ACS on the OMM, and has a regulatory role in LCFA oxidation. In this model, FAT/CD36 accepts LCFA from FABPc at the OMM which facilitates the delivery of LCFA to ACS to promote an increase in ACS enzymatic flux rates. This would result in an increased LCFA-CoA delivery to CPT-I, the transfer of LCFA into the mitochondrial matrix, and subsequently β-oxidation. This proposed mechanism-of-action for mitochondrial FAT/CD36 is similar to the proposed mechanism at the plasma membrane, where FAT/CD36 is thought to facilitate the transfer of LCFAs from albumin to the outer leaflet of the plasma membrane, thereby regulating LCFA transport into the cell (reviewed in [50,51]). CACT, carnitine acylcarnite translocase.

(fatty-acid-binding protein c) at the OMM, which would facilitate the delivery of LCFA to ACS allowing this enzyme to increase enzymatic flux rates. This would result in an increased LCFA-CoA delivery to CPT-I to increase the transfer of LCFA into the mitochondria, and subsequently increase rates of mitochondrial fatty-acid oxidation. In the context of this working model, CPT-I retains its essential role in LCFA transport as classically proposed [39,40]. However, unlike CPT-I, FAT/CD36 is not required for mitochondrial fatty-acid oxidation, as knocking out FAT/CD36 only reduced fatty-acid-supported respiration rates as opposed to completely ablating them. Therefore we propose that altering FAT/CD36 content at the mitochondrial level provides additional regulation that may be important for augmenting LCFA oxidation rates. This may be particularly important during exercise when the energetic demands from LCFA oxidation are increased substantially, a process that coincides with increases in mitochondrial FAT/CD36 [18,37,52].

CONCLUSION

Our data indicate that, in skeletal muscle, FAT/CD36 is located on the OMM and influences mitochondrial fatty-acid oxidation proximal to ACS. Impairments within mitochondrial fatty-acid oxidation and the dysregulation of lipid metabolism have been associated with various pathologies [53–55]. Therefore understanding the regulation of fatty-acid metabolism, including mitochondrial fatty-acid oxidation, may highlight potential therapeutic strategies.

AUTHOR CONTRIBUTION

Brennan Smith performed experiments and wrote the manuscript. Swati Jain and Aaron Dam performed experiments and edited the manuscript. Stéphanie Rimbaud, Joe Quadrilatero, Renée Ventura-Clapier and Arend Bonen interpreted data and edited the manuscript. Graham Holloway designed experiments, interpreted data and edited the manuscript.

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