A sensitive immunoassay for rat fatty acid translocase (CD36) using phage antibodies selected on cell transfectants: abundant presence of fatty acid translocase/CD36 in cardiac and red skeletal muscle and up-regulation in diabetes

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The rat membrane protein fatty acid translocase (FAT), which shows sequence similarity to human CD36 (a membrane protein supposedly involved in a variety of membrane processes), is implicated in the transport of long-chain fatty acids across cellular membranes. To set up an immunoassay for quantification of FAT in different tissues, we isolated a series of anti-FAT antibodies by panning a large naive phage antibody library on FAT-transfected H9c2 cells. All seven different phage antibody fragments isolated reacted specifically with FAT, and most likely recognize the same or closely located immunodominant sites on FAT, as a competitive monoclonal antibody (mAb) (CLB-IV7) completely blocked the binding of all these phage antibodies to cells. A sandwich ELISA was set up using mAb 131.4 (directed against purified CD36 from human platelets) as capture antibody and phage antibodies and anti-phage sera as detector. With this ELISA (sensitivity 0.05 μg/ml), the FAT content in isolated cardiomyocytes was found to be comparable with that of total heart (≈ 3 mg/g of protein), while liver tissue and endothelial cells were below the detection limit (< 0.1 mg of FAT/g of protein). During rat heart development, protein levels of FAT rose from 1.7 ± 0.7 mg/g of protein on the day before birth to 3.6 ± 0.4 mg/g of protein on day 70. Comparing control with streptozotocin-induced diabetic rats, a statistically significant ($P < 0.05$) 2–4-fold increase of FAT was seen in heart (from 4.2 ± 2.3 to 11.0 ± 5.7 mg/g of protein), soleus (from 0.6 ± 0.1 to 1.4 ± 0.5 mg/g of protein) and extensor digitorum longus (EDL) muscle (from 0.3 ± 0.1 to 1.2 ± 0.8 mg/g of protein). In addition, the FAT contents of each of these muscles were found to be of similar magnitude to the contents of cytoplasmic heart-type fatty-acid-binding protein in both diabetic rats and controls, supporting the suggested roles of these two proteins in cellular fatty acid metabolism. This is the first time phage display technology has been succesfully applied for direct selection, from a large naive antibody library, of antibodies that recognize selected membrane proteins in their natural context.

Key words: cell-based selection, ELISA, fatty-acid-binding protein, membrane protein, phage display.

INTRODUCTION

Long-chain fatty acids are important substrates for energy production in both heart and skeletal muscles [1,2]. Although diffusion is hypothesized to play a decisive role in the transport of fatty acids from the interstitial space into the cell interior [3], evidence is accumulating that membrane-associated and cytoplasmic proteins are involved in this process [4–6]. One of these is a 88 kDa membrane protein, firstly identified in rat adipocytes [7–9] and named (putative) fatty acid translocase (FAT). This rat protein shows substantial sequence similarity (85%) to human CD36, a membrane protein supposedly involved in a variety of membrane processes [10,11]. Whether FAT/CD36 functions as a true translocase has not yet been established. Alternatively, because of the presence of only a single or double transmembrane region [10], the protein may serve as ancillary glycoprotein to a membrane transporter, much like GP-70 functions with the monocarboxylate transporter [12] or CD98 with an amino acid transporter [13]. In trying to understand the precise physiological role of FAT/CD36, a reliable technique to measure the tissue and cellular contents of this protein is required. However, at present only immunohistochemical [14] and mRNA detection techniques [15] are available. An immunoassay to accurately quantify the amount of FAT protein is lacking, in part because no combination of antibodies has been found to produce a sandwich ELISA. For instance, some antibodies were found to recognize the native structure of FAT, while others are useful only in Western blotting, detecting denatured FAT only [16]. Raising antibodies against intact membrane proteins such as FAT has often shown to be problematic, in part because it is difficult to maintain the correct conformation of the protein in the absence of a membrane environment.

In order to obtain antibodies directed against FAT/CD36 in its natural conformation, we used the method of phage display (reviewed in [17]). By the use of *in vitro* enrichment procedures it has been shown that antigen-specific phage antibodies may be selected from large collections of phage antibodies derived from naive or synthetic antibody V-genes [17]. This method of making antibodies offers the advantage that, in principle, whole living cells expressing native antigen may also be used for the selection [18] and that large collections of antibodies can be tested (reviewed in [17,18]. Thus we isolated single-chain Fv (scFv)

Abbreviations used: FAT, fatty acid translocase; mAb, monoclonal antibody; human CD36, a membrane protein supposedly involved in a variety of membrane processes; scFv, single-chain Fv; FBS, foetal-bovine serum; CHO, Chinese-hamster ovary; c.f.u., colony-forming unit(s); TMB, tetramethylbenzidine; (H-)FABP, (rat-heart-type) fatty-acid-binding protein; EDL, extensor digitorum longus; fd, phage coat protein.

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antibody fragments from a large collection of phage antibody fragments displayed on the surface of filamentous phage [19] by selection on FAT-overexpressing cells. We first selected the repertoire comprising 6×10^9 human scFv fragments on FAT-overexpressing cell transfectants and, secondly, in a cell-based ELISA, screened the selected phage antibodies on antigen-positive and -negative counterparts to distinguish antigen-specific from irrelevant phage binders. Several selected phage antibodies that were proved to react specifically with FAT were subsequently used to develop a suitable sandwich-type ELISA for the quantification of FAT. With this immunoassay we measured the content of FAT in heart and skeletal muscles from control and streptozotocin-induced diabetic rats, and found that FAT is an abundant protein in muscle and that its muscular content is markedly elevated in diabetes, which is of interest in regard to known variations in fatty acid metabolism [20].

EXPERIMENTAL

Cells and tissues

H9c2(2-1) is a cell line derived from embryonic rat heart and was obtained from the American Type Culture Collection (A.T.C.C. CRL 1446). The cells were stably transfected with rat FAT by co-transfection, using the lipocarrier *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium sulphate ('DOTAP'; Boehringer, Mannheim, Germany), of the expression vector PSG5-FAT with a selectable marker gene carried by pMAMneo (ClonTech, Palo Alto, CA, U.S.A.) [21] and were cultured in Dulbecco's modified Eagle's medium (Gibco–BRL Life Technologies, Gaithersburg, MD, U.S.A.) supplemented with 9 % (v/v) fetal-bovine serum (FBS) (SEBAK, Aidenbach, Germany) and 0.05 % gentamycin. Cells were cultured under air/CO_2 (19:1, water-saturated). Northern-blot analysis of total RNA of 15 cell lines obtained after transfection showed different levels of FAT expression, varying from 0 to 50 % of the expression found in adult rat heart. Western-blot analysis using a monoclonal antibody (mAb), Mo25, raised against SDS-denatured human CD36 (a gift from Dr. D. E. Greenwalt, Human Genome Sciences, Rockville, MD, U.S.A.) confirmed the expression of FAT at the protein level. In addition, immunofluorescence labelling of living cells (impermeable to antibodies) revealed the presence of FAT on the plasma membrane. For the present study we selected two FAT-cell transfectants: 1A3 showing high levels of FAT mRNA expression, and 3B3, showing no measurable FAT mRNA expression [21]. Both cell lines show equal growth rates. Chinese-hamster ovary (CHO) cells transfected with somatostatin-receptor subtypes [22] were taken as additional negative control for screening.

To assess FAT protein contents of rat heart and liver, adult male Wistar rats (10 weeks) were killed and the heart and liver were perfused with Krebs–Henseleit buffer to remove blood. Tissues were then frozen in liquid nitrogen and stored at −80 °C for further analysis. In selected experiments tissues were not perfused. Changes in the heart during maturation were studied using hearts from Wistar rats at birth (day 0), and at 2, 6, 21 and 70 days. Foetal hearts were obtained from pregnant rats killed at day 21 of pregnancy (day −1). For comparison of the FAT content of isolated cardiomyocytes with that of total heart, cardiomyocytes were harvested from adult male Wistar or Lewis rats as described by Luiken et al. [6]. Endothelial cells were isolated from hearts of adult male Wistar or Lewis rats as described by Linssen et al. [23] and in the present study we used passages 1–3 as well as two immortalized cell lines designated RHEC-50 and RHEC-116.

To study the influence of experimental diabetes on FAT content in heart and skeletal muscles, six adult male Wistar rats of about 250 g were rendered diabetic by a single injection of 17.5 mg of streptozotocin in 1 ml of citrate buffer (70 mg/kg body weight), while six control animals received an injection with citrate buffer alone. After 3 weeks the animals were killed, and from each animal, heart, soleus, extensor digitorum longus (EDL) and liver were quickly removed and frozen in liquid nitrogen.

Before use, tissues were homogenized (1.5–10 % w/v) in SET buffer (10 mM Tris/2 mM EDTA/0.25 M sucrose, pH 7.4) using an Ultra-Turrax homogenizer (IKA Werke, Breisgau, Germany). The homogenates were then sonicated four times for 15 s each time (Soniprep 150; Sanyo Gallenkamp PLC, Leicester, U.K.; amplitude 12 μm). Isolated or cultured cells were also sonicated four times for 15 s each time.

Phage library

The phage library used in the selections was a subset of the library, with 6×10^9 clones, as described by Vaughan et al. [19], and was prepared from the naive V-gene pool of the B-lymphocytes from 43 non-immunized human donors. These V-gene segments were used to construct a very large repertoire of scFv fragments displayed on the surface of phage, which is known to be a source of mediate affinity antibodies for all antigens tested. The library was cloned into phagemid vector pCANTAB6, a derivative of pHEN1 [24], which enables analysis of selected phage antibodies both as phage displayed scFv fragments and as free scFv fragments (prepared by isopropyl thiogalactoside induction in a medium containing 0.1 % glucose), without subcloning.

Isolation of phage antibodies against FAT by selection on FAT-expressing cells

In each round of selection, approx. 2×10^{13} phage colony-forming units (c.f.u.) were used, pre-incubated for 30 min with 2 ml of PBS/2 % Marvel dried milk powder (Chivers and Sons Ltd., Coolock, Dublin, Ireland). For the first, crucial, selection round, this corresponds to approx. 3000 copies of each antibody clone. FAT-expressing 1A3 cells (see above) were grown to confluency, trypsin-treated and collected in PBS/10 % FBS. Pre-incubation was in PBS/2 % Marvel. The 1A3 cells were spun down (500 ***g***, 7 min), then resuspended using the phage-containing mix to a concentration of 7×10^6 cells/ml. After incubation for 2 h, unbound phage particles were removed by 12 washes with PBS/2 % Marvel, followed by washing with PBS. Washes were carried out by gently spinning the cells and removing the phage-containing supernatant. Cell-bound phages were eluted with 100 mM triethylamine for 10 min, collected, neutralized with 1 M Tris/HCl, pH 7.4, and prepared for infection of *Escherichia coli* TG1 cells. Phage needed for the subsequent selection round on 1A3 cells were obtained by rescue from these *E. coli* cells with M13K07 helper phage [24,25]. Before the third round of selection on 1A3 cells, the phage mix was depleted of irrelevant phage antibodies by pre-incubation with in total 20×10^6 FAT-negative cells (3B3). Following each selection round the ratio of eluted phage and input phage was determined via phage titration.

Whole-cell specificity test for phage antibodies and free antibody fragments

1A3 cells (12×10^6), 3B3 cells (12×10^6) and CHO cells transfected with somatostatin receptor subtype 2 (49×10^6) were resuspended

in PBS/2% Marvel after trypsin treatment and collection in PBS/10% FBS. Cells were apportioned to 50000 cells/well in a V-shaped 96-well microtitre plate for the whole-cell ELISA. The supernatant of overnight-grown *E. coli* TG1 cultures, containing either rescued phage antibodies or free antibody fragments in the supernatant, was incubated 1:1 in PBS/4% Marvel. The microtitre plate with eukaryotic cells was centrifuged (500 ***g***, 3 min) and supernatant was removed. The cell pellet was resuspended in 100 μl of the phage antibody or free antibody fragment mix and incubated for 1 h with mild shaking to avoid precipitation of cells. Following two washes with PBS, sheep anti-fd (phage coat protein) antibody (Pharmacia Biotech; 1:5000 in PBS/2% Marvel) was added for phage antibodies, or 9E10 anti-scFv mouse hybridoma supernatant (1:1 in PBS/2% Marvel) for free antibody fragments, followed by incubation for 1 h (with shaking). After two washes with PBS, peroxidase-labelled rabbit anti-goat IgG antibody (Dakopatts P-0449; Dako A/S, Copenhagen, Denmark; 1:2000 in PBS/2% Marvel) was added for phage antibodies, or peroxidase-labelled rabbit anti-mouse IgG antibodies (Dakopatts P-0260; 1:2000) for free antibody fragments, followed by incubation for 1 h. After one washing step, cells were transferred to a fresh microtitre plate (Falcon type 3912; Beckton–Dickinson, Oxnard, CA, U.S.A.) which was preblocked with PBS/2% Marvel, and the cells were washed for an additional three times. Finally, cells were resuspended in tetramethylbenzidine (TMB; Roche TMB Enzymatic Kit 0734845). After 20 min incubation, cells were spun (500 ***g***, 5 min) and 90 μl of the supernatant added to 100 μl of 5% H_2SO_4 in a fresh microtitre plate. A_{450} was measured with a microtitre plate reader (Titertek Multiscan Mk II).

Specificity ELISA of phage antibodies or free antibody fragments on recombinant proteins

A microtitre plate (Falcon type 3912) was coated overnight at 4 °C with either purified rat His_6-labelled FAT (a gift from Dr. N. A. Abumrad, Department of Physiology and Biophysics, State University of New York at Stony Brook, Stony Brook, NY, U.S.A.) in two concentrations (0.5 and 2 μg/ml), 100 μg/ml BSA (A4503 from Sigma), 2% Marvel, 10% FBS or 2 μg/ml recombinant rat-heart-type fatty-acid-binding protein (H-FABP) [26], each in 0.1 M sodium bicarbonate buffer, pH 9.4 (final vol. 100 μl/well). After washing with PBS/0.1% Tween-20, the plates were incubated with either free antibody fragments or phage antibodies (in PBS/2% Marvel/0.1% Tween-20) obtained from clones which produce phage antibodies reactive with 1A3 cells. After 2 h of incubation, plates were washed with PBS/0.1% Tween-20 and incubated either with 1:5000 sheep anti-fd (phage) or 1:1 mouse anti-scFv 9E10 hybridoma supernatant, each in PBS/2% Marvel/0.1% Tween-20. Incubation time was 1 h. Plates were washed again with PBS/0.1% Tween-20 and incubated as described above, but using PBS/2% Marvel/0.1% Tween-20 as incubation buffer. Following incubation for 1 h, plates were washed and TMB (Roche TMB Enzymatic Kit 0734845) was added. After 20 min incubation, 5% H_2SO_4 was added to stop the reaction and A_{450} was measured with a Titertek Multiscan Mk II microtitre plate reader.

DNA fingerprinting

The diversity of antibody fragments displayed on phages was determined by DNA fingerprint analyses of the antibody scFv fragment inserts as described in [27]. Clones selected after two and three rounds were randomly picked, and the full V_H-linker-V_L DNA (the DNA encoding for the variable part of the heavy and light chain of the antibody, coupled via a linker) inserts were amplified by PCR using oligonucleotides bordering the insert: LMB3 (5′-CAGGAAACAGCTATGAC-3′), which sits upstream from the pelB signal leader sequence, and fd-SEQ1 (5′-GAATTTTCTGTATGAGG-3′), which sits in the 5′ end of gene III. After digestion of these scFv inserts with the restriction endonuclease *Bst*NI (0.25 unit/μl), fragments were applied to a 3%-agarose gel (Gibco 15510-027). The gel was run in a wide mini-sub cell GT system (Bio-Rad, Hercules, CA, U.S.A.).

Antibody-capture ELISA for FAT

An ELISA of the antigen-capture type (sandwich ELISA) was developed using mAb 131.4 (IgG1 type, raised against CD36 purified from human platelets) [16] as catcher and purified phage as detector. Phage antibodies were produced and purified from *E. coli* TG1. In a poly(vinyl chloride) microtitre plate (Falcon type 3912), 200 ng/well mAb 131.4 was coated overnight at 4 °C in 0.1 M sodium bicarbonate buffer, pH 9.4). The wells then were rinsed five times with PBS containing 0.1% (w/v) BSA and 0.05% (v/v) Tween-20 (PBT). Thereafter wells were blocked for 30 min with 100 μl/well of PBS/2% Marvel. After five washing steps with PBT, 100 μl of a standard containing 0–1 μg/ml recombinant His_6-FAT in PBS/0.4%Triton X-100 was added per well. Tissue and cell samples were first diluted 1:1 in PBS/4% Triton X-100 and incubated at 37 °C for 1 h. After centrifugation for 2 min to remove cell debris, samples were diluted in PBS/0.4% Triton X-100 and added to the plate (100 μl/well). After incubation for 90 min, wells were washed five times with PBT. Then phage (2×10^{11} c.f.u./well in PBS/2% Marvel) were added and incubated for 90 min. After five washes with PBT, 1:5000-diluted sheep anti-fd in PBS/2% Marvel was added (100 μl/well). Incubation for 1 h was followed by five washes and addition of 1:1000-diluted peroxidase-labelled rabbit anti-goat IgG (Dakopatts P-0449) in PBS/2% Marvel. After 1 h incubation and five washes, plates were developed with 100 μl of 1 mmol/l TMB/well. The reaction was stopped after 10 min with 50 μl 5% H_2SO_4/well and A_{450} was measured using a Titertek Multiscan MkII microplate reader.

In selected experiments (direct ELISA on coated His_6-FAT) we also compared CLB-IV7, raised against human CD36 by immunizing a BALB/c mouse with human monocytes (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) with phage antibodies.

Other analytical techniques

The tissue content of cytoplasmic H-FABP was measured with an ELISA of the antigen-capture type (sandwich ELISA), using rabbit IgG antibodies directed against purified H-FABP and the streptavidin–biotin detection system [28]. Tissue samples were homogenized (5%, w/v) in ice-cold PBS, pH 7.4, and centrifuged at 15000 ***g*** for 15 min at 4 °C to remove cell debris. The FABP content was determined in several dilutions of the supernatant.

Protein determination

Total protein content of tissue and cell homogenates was determined with the micro-bicinchoninic acid ('BCA') kit (Protein Assay No. 23235; Pierce, Rockford, IL, U.S.A) using BSA as standard [29].

Statistics

Differences in FAT contents of muscles from control and diabetic animals were analysed by the non-parametric Mann–Whitney test. Data are presented as means ± S.D. for the indicated number of animals.

RESULTS

Selection of phage antibodies

FAT-positive cells (1A3) were incubated with 2×10^{13} phage (input). After removal of unbound phage by a series of washes, bound phage (output) were eluted and used for re-infection of *E. coli* TG1 in order to obtain phage for further selection rounds. From the second to the third round of selection, the output/input ratio of phage markedly increased, i.e., from 1.33×10^{-5} to 1.41×10^{-3}. As this enrichment appeared to be sufficient for the isolation of specific clones (see below), no further rounds were performed. After each selection round, 45 clones were picked from which either phage antibodies or free antibody fragments were produced.

Specificity was first examined by whole-cell ELISA with the phage antibodies. 1A3 cells (10^5/well) were tested as FAT-positive cells and 3B3 (10^5/well) and CHO (10^6/well) as FAT-negative cells. Two rounds of selection gave seven specific clones out of 45 (16%) and, after the third round, 37 clones out of 45 (82%) showed specific binding to FAT-expressing cells. DNA fingerprinting of 24 out of the 45 clones picked after the third round of selection indicated 20 different sequence patterns (Figure 1). Only clones 1, 3 and 14 had the same pattern of scFv DNA fragments, as did clones 5 and 11, and clones 7 and 22.

For logistic reasons, we selected 14 out of 37 positive clones, each having a different DNA pattern. From these clones, free antibody fragments were produced, and these were tested for specificity in a whole-cell ELISA on 1A3, 3B3 and CHO cells. As shown in Figure 2, antibody fragments produced from 12 clones bound specifically to 1A3 cells. Antibody fragments from only one clone (number 8) bound to both 1A3 and 3B3 cells (and

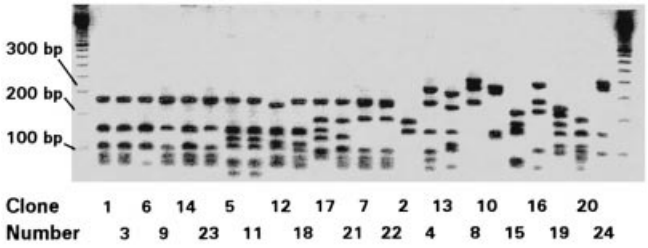

Figure 1 DNA fingerprint analysis of selected scFv antibody fragments

*Bst*N1 fingerprint analysis of scFv antibody fragment inserts (V_H-linker-V_L) of 24 clones picked after the third round of selection on FAT-overexpressing 1A3 cells.

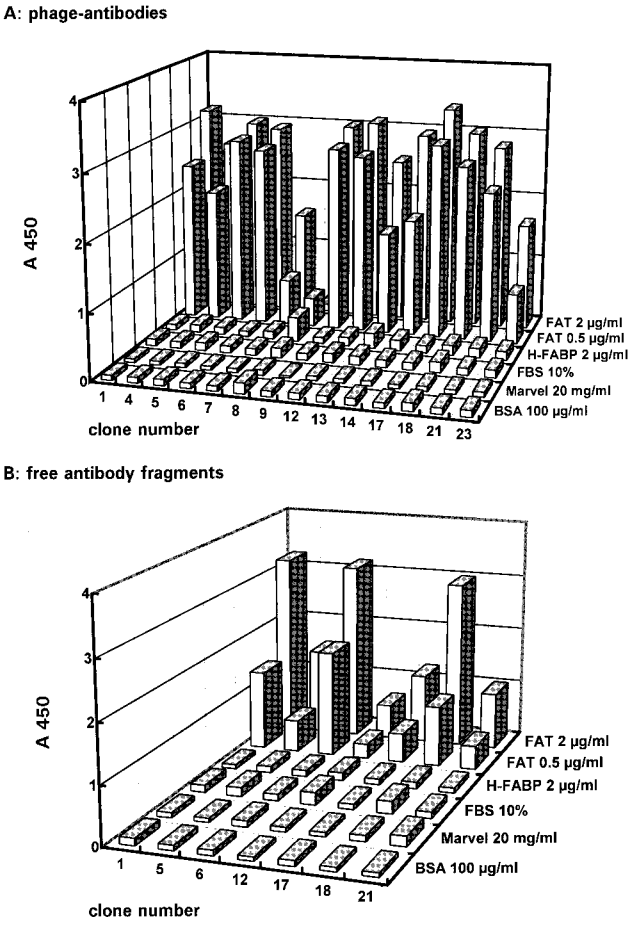

Figure 3 Specificity check of phage and free antibody fragments on recombinant His_6-FAT

Specificity ELISA with phage antibodies (**A**) or a selected set of scFvs (**B**) produced from clones picked after three rounds of selection on FAT-overexpressing 1A3 cells. Tests were carried out with immobilized recombinant rat His_6-FAT, BSA, Marvel, FBS and recombinant H-FABP.

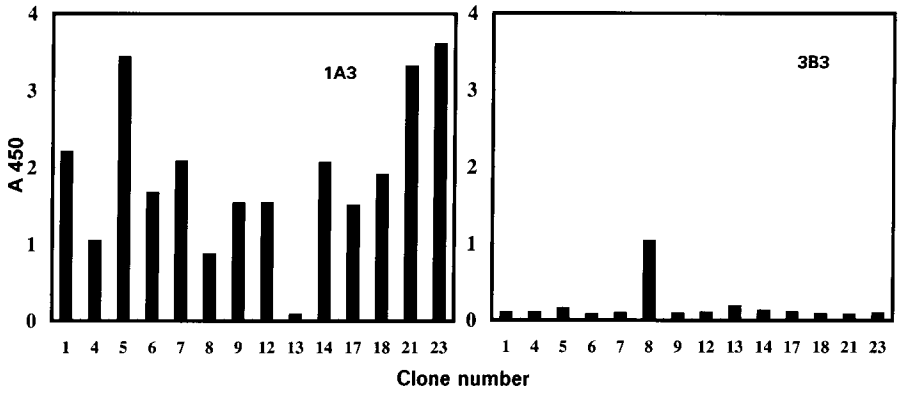

Figure 2 Specificity check of free antibody fragments on FAT positive (1A3) and FAT negative (3B3) cells

Whole-cell ELISA with free antibody fragments produced from clones picked after the third round of selection on FAT-overexpressing 1A3 cells. Fragments were tested on 1A3 cells (left panel) and FAT-negative 3B3 cells (right panel). Clone numbers correspond to those in Figure 1.

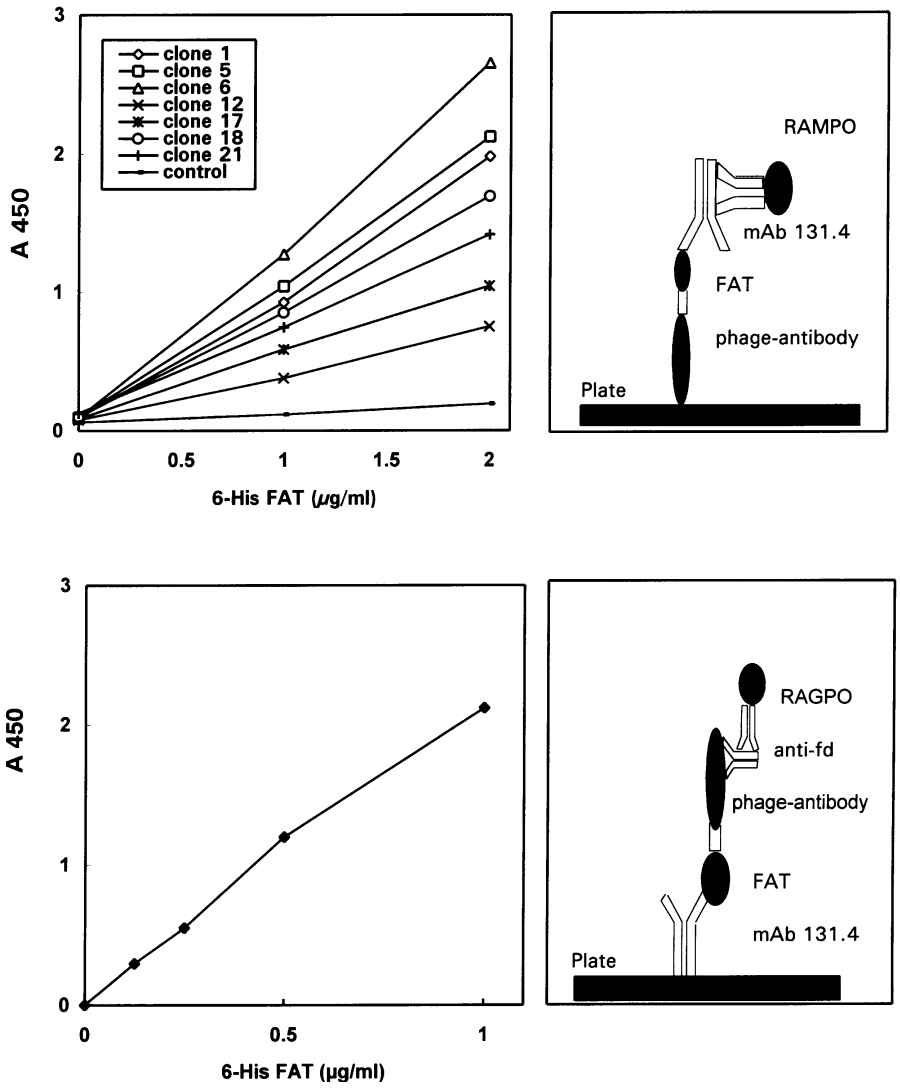

Figure 4 ELISA set up for FAT/CD36

Calibration curves for two assay formats are given. Upper panels: coated phage antibodies as capture antibody and mAb 131.4 as detector antibody. Lower panels: mAb 131.4 as capture antibody and phage 6 as detector antibody. Clone numbers correspond to those in Figures 1 and 2. Results from representative experiments are shown. 6-His FAT, His_6-tagged FAT; RAMPO, rabbit anti-mouse peroxidase; RAGPO, rabbit anti-goat peroxidase; anti-fd, sheep anti-fd antiserum.

therefore are not specific), whereas fragments from another clone (number 13) showed no signal when incubated with either cell type.

The specificity of the clones was also checked as phage antibodies on purified His_6-FAT in an antibody-capture ELISA. For this, a microtitre plate was coated with 0.5 and 2 μg/ml recombinant FAT and, for comparison, also with 100 μg/ml BSA, 20 mg/ml Marvel, 10% FBS or 2 μg/ml cytoplasmic H-FABP. Results of the ELISA are shown in Figure 3(A). All phages bound specifically to FAT, while phages 8 and 13 also showed low binding to H-FABP. The scFvs of seven selected phages were tested in the same ELISA set-up as that described above (Figure 3B). These scFvs showed each a concentration-dependent binding to FAT, while binding to the other antigens was virtually absent (low signal on FBS).

In order to characterize the selected phage further, binding of the phage antibodies in a whole-cell ELISA was studied in the presence of the anti-(human CD36) mAb CLB-IV7. For each of the seven selected phage antibodies the binding to FAT-expressing cells was blocked in the presence of CLB-IV7. This suggests competition for antibody-binding sites, because the selected phage and antibody CLB-IV7 each recognize either the same epitope or epitopes in an area of the protein molecule causing steric hindrance upon antibody binding.

Development of a sandwich-type ELISA for FAT

Because the selected phage antibodies were found to be directed against the same epitope or closely related epitopes on the FAT protein molecule, it was not possible to develop an ELISA of the antibody-capture type (sandwich ELISA) using a combination of two of the selected phage antibodies. Also, the mAb CLB-IV7 did not recognize the recombinant His_6-FAT. Therefore, we used another mAb to develop a sandwich-type ELISA involving phage antibodies. This antibody, coded 131.4, was raised to human CD36 purified from platelets [16] and did not block the

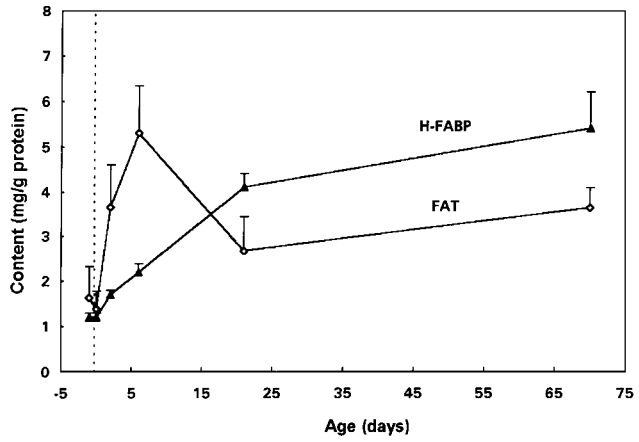

Figure 5 FAT and H-FABP protein contents of rat heart during development

Data represent means ± S.D. for five animals. ▲, H-FABP (mg/g of protein); ◇, FAT (mg/g of protein).

Table 1 FAT and H-FABP protein content of adult rat muscles

The contents of FAT and H-FABP were measured in non-perfused heart, skeletal muscles and liver obtained from adult control and streptozotocin-induced diabetic rats. Both FAT and H-FABP values were determined via an ELISA specific for FAT or H-FABP respectively. Data are expressed in mg per g of total protein and are means ± S.D. for six animals. *Significantly different from control ($P < 0.05$).

	Content (mg/g of protein)			
	FAT		H-FABP	
Tissue	Control	Diabetic	Control	Diabetic
Heart	4.2 ± 2.3	11.0 ± 5.7*	5.7 ± 1.1	7.4 ± 2.4
Soleus	0.6 ± 0.1	1.4 ± 0.5*	2.6 ± 0.2	4.8 ± 0.8*
EDL	0.3 ± 0.1	1.2 ± 0.8*	0.5 ± 0.1	0.8 ± 0.2*
Liver	0.2 ± 0.3	0.2 ± 0.1	< 0.1	< 0.1

binding of the phage antibodies to whole cells (results not shown). Subsequently a sandwich-type ELISA was set up using purified phages as capture antibody, while mAb 131.4 was used as detector antibody. Figure 4 (upper left panel) shows that mAb 131.4 in combination with any of the phage gave a useful calibration curve with comparable sensitivity of the assay. However, when 0.4 % Triton X-100 was included in the dilution buffer, absorbances decreased more then 50 %, indicating that bound phage were released from the wells. Since for the determination of FAT in tissue preparations Triton X-100 is required to release FAT from membranes, we decided to use the mAb 131.4 as capture antibody, bound to the microtitre plate via electrostatic interaction in 0.1 M sodium bicarbonate buffer, pH 9.4. After binding of FAT, phage antibodies prepared from clone 6 were used as detector. This set up also gave a linear calibration curve (from 0 to 1 μg/ml FAT), as is seen in Figure 4 (lower left panel); phage antibodies prepared from other clones gave results similar to those prepared from clone 6 (results not shown). With this approach, Triton X-100 treatment did not interfere in the assay procedure, making this set-up the preferred sandwich. The lower limit of detection of this assay was 0.05 μg/ml (blank + three times the S.D.).

Application of the FAT sandwich ELISA for quantification of FAT in rat tissues

The developed ELISA was applied for determination of the contents of FAT in various rat tissues and isolated cells. All tissue and cell samples were treated with Triton X-100 (final concn. 2 %) to solubilize FAT. Addition of 0.5, 1, 2 and 4 % Triton X-100 showed that final concentrations higher than 2 % gave no further increase in FAT release. The ELISA blank was not influenced by the presence of Triton X-100. To avoid matrix effects, samples were diluted at least 5-fold with PBS.

The FAT-transfected and FAT-expressing cells (1A3) contained 28.3 mg of FAT/g of protein, while the non-expressing cells (3B3) and the original non-transfected H9c2 cell line showed no detectable amount of FAT (< 0.1 mg/g protein). Isolated cardiomyocytes (2.9 ± 0.4 mg/g of protein) showed FAT levels comparable with perfused total heart (3.1 ± 1.2 mg/g protein; mean ± S.D., $n = 3$). The non-perfused total heart also showed comparable FAT content (4.2 ± 2.3 mg/g protein; mean ± S.D., $n = 6$). Microvascular endothelial cells isolated from adult heart and two immortalized rat heart endothelial cell lines (RHEC-50 and RHEC-116) [23] showed no detectable amounts of FAT (< 0.1 mg/g of protein). Finally, the FAT content of non-perfused liver (0.2 ± 0.3 mg/g of protein; mean ± S.D., $n = 6$) tended to be higher than that of perfused liver (< 0.1 mg/g of protein). This latter difference most likely is due to the presence of platelet CD36 in the non-perfused liver.

During rat heart development, the FAT protein content increases from 1.7 ± 0.7 mg/g of protein before birth (day −1) to 3.6 ± 0.4 mg/g of protein at day 70 (Figure 5). For comparison we also measured the contents of cytoplasmic H-FABP during cardiac development, to find this protein to increase from 1.2 ± 0.1 mg/g of protein (day −1) to 5.4 ± 0.8 mg/g of protein at day 70 (Figure 5).

Because we previously found [30] that the expression of FAT (mRNA level) is up-regulated in the heart and skeletal msucles of streptozotocin-induced diabetic rats, we examined FAT protein contents in this condition and, for comparison, also studied the tissue contents of cytoplasmic H-FABP. The FAT content of the diabetic heart was 2–3-fold higher than that of non-diabetic control animals (Table 1). Oxidative soleus and glycolytic EDL muscles showed markedly lower FAT contents than cardiac muscle, but a similar 2–3-fold upregulation in the diabetic animals (Table 1). The muscle contents of H-FABP also decreased from heart to soleus to EDL from both control and diabetic rats, but experimental diabetes elicited a statistically significant increase of H-FABP content only in soleus and EDL muscles (Table 1.)

DISCUSSION

Selection of phage antibodies

The use of very large naive antibody libraries has permitted the isolation of antibodies to many different antigens [18], but in most reported cases a source of highly purified antigen was used for the *in vitro* selection procedure. There are very few examples of selection of naive phage antibody libraries on cell transfectants, with the aim to generate antibodies to the cell-surface marker that has been overexpressed on the cell surface (reviewed in [18]). The reason for this is that the use of impure antigen preparations with such naïve libraries is significantly more difficult, owing to the problem of limited amount of target antigen present in the mixture and of enrichment of phage antibodies specific for non-relevant antigens. Depletion and/or subtraction methods, com-

petitive elution with an antibody or the antigen itself (when available) or selection by alternating between different sources of antigen could all be used to drive the selection towards the cell-surface antigen of interest (see [18] and references therein). In our approach, we isolated 14 different FAT-specific phage antibodies by two or three rounds of panning on H9c2 cell transfectants, with (in round 3) only one depletion on non-transfected cells to remove phage antibodies binding to irrelevant targets. Most antibodies recognize the immunodominant epitope on FAT, located in a region that also interacts with other molecules (see below).

The reasons why these selections have worked so efficiently, with a minimal requirement of depletion to remove irrelevant phage antibodies, and with a very fast enrichment of antigen-specific phage antibodies (one round faster than seen with other cell transfectants; H. R. Hoogenboom, J. T. Lutgerink and M. M. A. L. Pelsers, unpublished work) are unclear. Strikingly, selections using the human FAT homologue CD36, in a very similar way as described here for FAT, albeit using CHO-K1 cell transfectants, also yielded specific phage antibodies in 3 selection rounds (H. R. Hoogenboom, J. T. Lutgerink and M. M. A. L. Pelsers, unpublished work). These data may be explained by the highly accessible nature of the FAT/CD36 structure to other biomolecules. It has been hypothesized that FAT/CD36 contains one large extracellular domain [10,31] which contains the major site of interaction with effectors, i.e. binding of oxidized low-density lipoprotein to monocytes, binding of *Plasmodium falciparum*-infected erythrocytes to endothelial cells, phagocytosis of apoptotic polymorphonuclear monocytes by cells transfected with CD36, and CD36-dependent platelet activation [32–35]. This epitope is overlapping with the binding site for CLB-1V7, and is also the 'selection'-dominant phage antibody epitope. Therefore this epitope area of the FAT/CD36 molecule must be well accessible for interaction with other proteins, including phage antibodies. Its high-level expression may ensure the rapid selection of epitope-specific phage antibodies, possible aided by the glycosylation of FAT/CD36, shielding other epitopes on this or other membrane receptors. Indeed, only very few phage antibodies specific for other H9c2-membranous antigens are recovered.

Development of a sandwich-type ELISA for FAT.

All phage antibodies to FAT-expressing cells unfortunately recognized one and the same, or overlapping, epitope. The mAb 131.4, which is directed against purified native CD36 from human blood platelets and cross-reacts with the same region on rat FAT, recognizes a different epitope on FAT than all of the selected phage antibodies. The combination of mAb 131.4 as capture antibody and phage as detector antibody to measure FAT derived from (cardiac) muscle cells was therefore used to set up an ELISA for measurement of solubilized FAT. The test's calibration curve is linear from the lower limit of detection (0.05 μg/ml) to 1 μg/ml, while samples should be diluted at least 5-fold to avoid matrix effects. Thus the detection limit for tissue samples was 0.25 μg/ml. It should be noted that the mAb CLB-IVC7 did not recognize the recombinant His_6-FAT, so that the combination with mAb 131.4 could not be used to set up an ELISA.

Applying the sandwich-type ELISA for FAT quantification in rat tissue homogenates

Until now, reports about FAT/CD36 contents of heart and skeletal muscles gave only qualitative differences. Applying immunohistochemistry, Greenwalt et al. [14] found FAT expression in capillary endothelial cells of murine adipose tissue and cardiac- and skeletal-muscle tissue, while the cardiac- and skeletal-muscle cells themselves were found to be CD36-negative, as was liver tissue. However, in histological autoradiography studies using a radiolabelled ligand that specifically binds to FAT, Tanaka and Kawamura [36] demonstrated the presence of FAT in cardiac myocytes. However, in addition, Van Nieuwenhoven and co-workers [15,21] did show FAT expression in cardiomyocytes, which now is in line with the measurable FAT protein levels in the present study. In contrast with the findings of Greenwalt et al. [14], the endothelial cells obtained from adult rat heart showed no detectable FAT content, which is in agreement with our previously published FAT mRNA data [15,30]. Because we used isolated endothelial cells and immortalized cell lines, the discrepancy might be due to down-regulation of FAT during cell culture.

We did not find detectable levels of FAT in perfused liver tissue, which is in agreement with previous studies in which no FAT-mRNA levels could be detected in liver tissue [9,15]. However, in non-perfused liver tissue we found low amounts of FAT, and this can be explained by the fact that non-perfused tissues contain platelets, which carry relatively high levels of CD36 [37].

In the second application of the sandwich-type ELISA, we examined the level of rat heart FAT from neonatal to adult and we noticed that FAT levels increase during development. In a previous study [15], cardiac mRNA levels of FAT also showed an increase from neonatal to adult. Our findings therefore affirm the notion that FAT is up-regulated during heart development, possibly adjusting to the increased amount of long-chain fatty acids to be transported across the cellular membrane necessary for energy conversion [1,38]. Since it seems that the rate of increase in FAT at the protein level is somewhat quicker than at the mRNA level, a regulation at the level of translation of FAT is likely.

In streptozotocin-induced diabetic rats we noticed not only in heart, but also in the oxidative soleus muscle as well as the glycolytic EDL muscle, a statistically significant ($P < 0.05$) higher FAT level as compared with control muscles. This can be explained by the fact that, under diabetic conditions, the muscles rely more on fatty acid oxidation for ATP formation than do control animals [39]. A striking and novel finding of our study is that the FAT protein contents of rat cardiac and skeletal muscles are of a similar order of magnitude as those of cytoplasmic H-FABP, which is known to be an abundant protein in muscles with a high fatty acid metabolism [40]. Furthermore, we have now established, for the first time at the protein level, that both FAT and H-FABP show a similar muscle tissue distribution (heart $\gg$ soleus muscle $\gg$ EDL muscle) and a similar cellular localization in the heart, and are up-regulated during development (heart) and upon induction of experimental diabetes (skeletal muscles) in the same way. These data are in line with the reported protein–protein interaction between FAT and H-FABP [41] and, taken together, further support the role for FAT in the cellular uptake of long-chain fatty acids by muscle cells.

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