Identification of a novel integral plasma membrane protein induced during adipocyte differentiation

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Adipocyte differentiation is co-ordinately regulated by several transcription factors and is accompanied by changes in the expression of a variety of genes. Using mRNA differential display analysis, we have isolated a novel mRNA, DD16, specifically induced during the course of adipocyte differentiation. DD16 mRNAs are present in several tissues, but among the tissues tested, a remarkably higher level of expression was found in white adipose tissue. The DD16 cDNA encoded a polypeptide of 415 amino acids containing a single N-glycosylation site and an N-terminal hydrophobic stretch of 19 amino acids forming a transmembrane segment, indicating that DD16 is a glycosylated membrane-bound protein. Polyclonal antibodies raised against the DD16 peptide detected immunoreactive DD16 in membrane fractions, notably the plasma membrane. Association of DD16 with the plasma membrane was further confirmed by biotinylation studies of cell surface proteins, suggesting that DD16 is an integral plasma membrane protein. Therefore we propose to give DD16 the name APMAP (Adipocyte Plasma Membrane-Associated Protein). Although the biological function of this polypeptide is presently unknown, our data suggest that APMAP may function as a novel protein involved in the cross-talk of mature adipocytes with the environment.

Key words: adipose tissue, differential display, 3T3-L1.

INTRODUCTION

White adipose tissue is the major energy reserve in higher eukaryotes and is composed mainly of adipocyte cells that store energy in the form of triacylglycerols in times of nutritional excess, and release free fatty acids during nutritional deprivation. In order to perform these functions, mature adipocytes express a large set of enzymes and regulatory proteins needed to carry out both *de novo* lipogenesis and lipolysis [1]. Furthermore, mature adipocytes are capable of exerting regulatory functions through the secretion of paracrine and endocrine factors [2–6]. The quantity of white adipose tissue varies widely in mammals and is dependent upon genetic background and environmental factors, including diet and physical activity. At the cellular level, increased body fat is a result of an increase in both cell size and number, and animal studies strongly suggest that the adipose tissue depots contain preadipocytes with the potential to differentiate into mature adipocytes throughout the lifespan of the animal [7,8].

Studies on adipocyte differentiation have been greatly facilitated by the availability of established immortalized preadipocyte cell lines such as 3T3-L1 and 3T3-F442A cells [9,10]. Under appropriate hormonal control, these cell lines differentiate into adipocytes with many characteristics of adipose cells found *in vivo*, and extensive studies indicate that these cells represent faithful models for adipocyte differentiation [11–13]. At the molecular level, differentiation of preadipocytes to mature adipocytes results in differential expression of a variety of genes. These include genes encoding proteins involved in lipogenesis and lipolysis [14–17], cytoskeletal and extracellular structure [18–20], cell cycle control [21,22], transcriptional control [23–29], intracellular transport [30,31], intracellular signalling [32–36], as well as a large number of secreted proteins [2–6,30,37,38] and several proteins of unknown function [39–41].

In the present study we have characterized a novel mRNA encoding a putative transmembrane protein that is strongly induced during the course of adipocyte differentiation. The mRNA was initially cloned by mRNA differential display analysis of differentiating 3T3-L1 cells. Subsequently, the up-regulation of the mRNA was confirmed in differentiating primary rat preadipocyte cultures, which strongly suggests that it is a novel marker for adipocyte differentiation. Several analyses of the polypeptide encoded by this mRNA indicate that it is an N-glycosylated integral plasma membrane-associated protein. The localization of the putative N-glycosylation site indicates that the protein contains a large extracellular domain and a considerably smaller intracellular domain. Taken together with the higher expression found in adipocytes compared with other tissues tested, our experiments suggest that this novel protein may play a role in signalling from the cell surface of mature adipocytes.

EXPERIMENTAL

Cell culture and hormonal treatment

Reagents were purchased from Gibco BRL unless otherwise stated. Murine 3T3-L1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM D-glucose and 4 mM glutamax supplemented with 10 % fetal bovine serum,

Abbreviations used: 36B4, acidic ribosomal phosphoprotein PO; aP2, adipocyte P2; APMAP, adipocyte plasma membrane-associated protein; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; ER, endoplasmic reticulum; G_{β}, G-protein β-subunit; GLUT4, glucose transporter isoform 4; HDM, high density microsomes; HRP, horseradish peroxidase; KRH, Krebs-Ringer Hepes; LDM, low density microsomes; LPL, lipoprotein lipase; NHS-LC-biotin, sulphosuccinimidyl-6-biotinamidohexanoate; PPARγ, peroxisome proliferator activated receptor γ; RACE, rapid amplification of cDNA ends; SHB, sucrose holding buffer; TNFα, tumour necrosis factor α; WRS, tryptophanyl tRNA synthetase.

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100 units/ml penicillin and 100 μg/ml streptomycin. Cells were grown to confluence and induced to differentiate two days post-confluence with media containing 1 μM dexamethasone (Sigma), 10 μg/ml human insulin (Novo Nordisk A/S) and 1 μM BRL49653 (Novo Nordisk A/S). For hormonal treatment, cells were differentiated for 7 days with dexamethasone and insulin and subsequently treated with 25 ng/ml tumour necrosis factor α (TNFα; Sigma) for various time intervals.

Primary preadipocytes were isolated as described previously [42]. Briefly, for each isolation 20 Sprague–Dawley male rats (Moellegaard Breeding and Research Center A/S) were killed by cervical dislocation. Epididymal fat pads were carefully dissected to avoid connective tissue and blood vessels and minced with scissors to give fragments of a maximum 1 mm in size. Subsequently, the tissue was digested in Krebs-Ringer Hepes (KRH) buffer (120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.6 mM $CaCl_2$, 25 mM Hepes, pH 7.4) supplemented with 1 mg/ml collagenase, 2 mM glucose and 3.5 % (w/v) BSA for 45–55 min at 37 °C in a shaking incubator at 180 strokes/min. Digested tissue was filtered through two layers of autoclaved gauze and the cell suspension was washed successively with 10 ml of KRH buffer supplemented with 1 % (w/v) BSA, 0.2 μM adenosine and 2 mM glucose. Between each wash, the preadipocytes were allowed to stand at room temperature for 1–2 min to assist in the separation from adipocytes before being washed in DMEM containing 25 mM D-glucose and 4 mM glutamax supplemented with 10 % heat-inactivated fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin and centrifuged at 1000 ***g*** for 20 min. The preadipocyte-enriched pellet was resuspended in 1 ml of animal erythrocyte lysis buffer (154 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) and filtered through a 70 μm nylon cell strainer (Falcon) followed by a 30 μm filter (Sefar AG). After filtration, the cell suspension was centrifuged at 500 ***g*** for 10 min and the preadipocyte pellet was resuspended in DMEM containing 25 mM D-glucose and 4 mM glutamax supplemented as described above and seeded at a density of 2×10^5 cells per dish. Primary cells were cultured at 37 °C in a humidified atmosphere containing 5 % CO_2 in air. After 2 days of culture, the medium was replaced with differentiation medium [DMEM containing 5.55 mM D-glucose and 4 mM glutamax/Ham's F12 (1:1 v/v) supplemented with 15 mM $NaHCO_3$, 15 mM Hepes, 33 μM biotin (Sigma), 17 μM panthotenate (Sigma), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 10 μg/ml transferrin (Sigma), 1 μM insulin, 200 pM 3,3′,5-tri-iodothyronine (T_3; Sigma) and 1 μM BRL49653]. The day of induction of differentiation was referred to as day 0.

Differential display analysis

mRNA differential display was performed essentially as described [43]. Briefly, total cellular RNA was isolated from 3T3-L1 cells harvested 2 days post-confluence and after 1 or 7 days treatment with dexamethasone, insulin and BRL49653 using RNAzol (AMS Biotechnology) according to the manufacturer's instructions. To remove traces of DNA, 50 μg of total RNA was treated with 3 units of RNase-free RQ1 DNase (Promega) and purified on Microcon 100 columns (Amicon) according to the manufacturer's instructions. Subsequently, three individual reverse transcription reactions were performed on RNA from each time point using one of three different 1 bp-anchored 3′-oligo(dT) primers with a *Hin*dIII recognition sequence (5′-AAGCTTT_{11}G-3′, 5′-AAGCTTT_{11}A-3′, or 5′-AAGCTTT_{11}C-3′) in a reaction buffer containing 50 mM Tris/HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 μM 1 bp-anchored 3′-oligo(dT) primer, 200 units of Superscript II reverse transcriptase (Gibco BRL), 20 μM dNTP and 2 μg of DNase-treated RNA in a total volume of 20 μl for 60 min. Differential display PCR reactions were performed using the same 1 bp-anchored 3′-oligo(dT) primer together with one of a small collection of arbitrarily designed 5′-oligos of 13 bp in length, including a *Hin*dIII recognition sequence (5′-AAGCTT-7 random nucleotides-3′). The sequence of the 5′-arbitrary oligonucleotide that gave a DD16 PCR product was 5′-AAGCTTCTGACAC-3′. A portion (2 μl) of the reverse transcribed cDNA was used for each PCR reaction containing 10 mM Tris/HCl, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001 % gelatin, 2 μM dNTP, 1 μCi of [α-^{33}P]dATP (Amersham Pharmacia Biotech), 0.2 μM 5′-arbitrary oligo, 1 μM 1 bp-anchored 3′-oligo(dT) primer, 1 unit of Taq2000 (Stratagene) and 0.22 μg anti-Taq antibody (Clontech) in a total volume of 20 μl. Parameters for PCR were: 40 cycles of denaturing at 94 °C for 15 s, annealing at 40 °C for 2 min and extension at 72 °C for 30 s. This was followed by 72 °C for 5 min. A portion (0.6 μl) of the PCR reactions was loaded on to a 6 % sequencing gel ('Cast-away'; Stratagene) and PCR fragments were visualized by exposing the dried sequencing gel to X-ray film. Differentially amplified bands were excised and the DNA was eluted by boiling the gel slices in 100 μl of water for 15 min. The eluted DNA fragments were re-amplified using the same primer pair and PCR conditions as for the differential display PCR, and cloned into the TOPO TA cloning vector (Invitrogen). The sequence of the cloned DNA fragment was determined using an ABI 377 (PerkinElmer).

Northern-blot analysis

Total RNA was isolated from 3T3-L1 cells using RNAzol according to the manufacturer's instructions. A portion (20 μg) of RNA was fractionated on a denaturing gel containing 1 % agarose, 20 mM Mops, 5 mM sodium acetate, 6 % formaldehyde and 1 mM EDTA, transferred to a Hybond N^+ membrane (Amersham Pharmacia Biotech) by capillary blotting and immobilized by UV crosslinking. cDNA encoding DD16 was labelled with the Prime It kit (Stratagene) using [α-^{32}P]dATP (Amersham Pharmacia Biotech), hybridized using Express Hyb (Clontech) according to the manufacturer's instructions, and the results were visualized by autoradiography.

cDNA cloning

Using a cDNA library from murine brain (Marathon-Ready; Clontech), a full length DD16 cDNA was obtained by 5′-rapid amplification of cDNA ends (RACE) using the gene-specific primer 5′-GTAGAGGGTGGGAGAGCACACCTTTTCTG-3′ according to the manufacturer's instructions. Eight individual clones were obtained and sequenced using an ABI 377 sequencer (PerkinElmer).

Animal studies

At 10 weeks of age, male C57BL mice were divided into groups of three animals and housed in a room controlled for temperature, humidity and light for 1 week prior to the experiment. Fasted animals were deprived of food for 16 h. Animals were killed by decapitation and the liver and white adipose tissue were removed and immediately frozen in liquid N_2. RNA was isolated by RNAzol (AMS Biotechnology) according to the manufacturer's instructions. All animal experiments were conducted in accordance with Danish law.

Quantitative PCR

Total RNA isolated from both mouse tissues and differentiating primary rat preadipocytes were DNase treated, and reverse transcription reactions were performed using Superscript II reverse transcriptase (Gibco BRL) according to the manufacturer's instructions. DD16 mRNA expression was analysed using real-time fluorescent detection using a Lightcycler (Roche) and the following primer combinations 5′-TTGGTGTTCTGCATCCAAATACGA-3′ and 5′-GGTCTCCCACAGGTAGGTTCATC-3′. Adipocyte P2 (aP2) and lipoprotein lipase (LPL) mRNA expression in differentiating rat preadipocytes were analysed using the primers 5′-ATGCCTTTGTGGGAACCTGG-3′ and 5′-CCCAGTTTGAAGGAAATCTCGG-3′ for aP2 and 5′-GCCAAGAGAAGCAGCAAGATGT-3′ and 5′-GGCAGGGTGAAGGGAATGTT-3′ for LPL. The DD16 mRNA expression levels in adipose tissue and livers of mice were normalized to the expression level of 18S RNA according to the manufacturer's instructions (Applied Biosystems). The DD16 mRNA expression levels in differentiating preadipocytes were normalized to the expression levels of acidic ribosomal phosphoprotein PO (36B4) using the primers 5′-TAAAGACTGGAGACAAGGTGGGAG-3′ and 5′-AGAAAGCGAGAGTGCAGGGC-3′.

In vitro transcription and translation of DD16 cDNA

DD16 was *in vitro* transcribed and translated using a TNT coupled system (Promega) following the manufacturer's instructions. The translated protein was labelled with [^{35}S]-methionine (1000 Ci/mmol; Amersham Pharmacia Biotech), separated by SDS/PAGE using 4–12 % NuPAGE gels (Novex) and visualized by autoradiography.

Preparation of antibodies against DD16 and Western-blot analysis

A polyclonal rabbit antiserum was raised against the amino terminus of DD16 using a synthetic peptide containing residues 1–14 of the DD16 sequence. The peptide was conjugated to ovalbumin using the coupling agent 1-ethyl-3(3-dimethylaminopropyl)carbodiimide ('EDAC'). Immunization of rabbits was performed by subcutaneous injection of the hapten–carrier complex emulsified in Freund's complete adjuvant followed by three booster injections of the antigen in incomplete Freund's adjuvant.

For Western blotting, proteins were separated by SDS/PAGE using 4–12 % NuPAGE gels (Novex) and transferred to nitrocellulose membranes (Kem-En-Tec) using a semi-dry blotter (Kem-En-Tec). The membranes were incubated in blocking buffer [PBS containing 5 % (w/v) non-fat dry milk and 0.05 % Tween 20] for 2 h and incubated with rabbit anti-DD16 serum (diluted 1:1000 in blocking buffer) for 3 h. After incubation, the membrane was washed four times in blocking buffer and incubated with horseradish peroxidase (HRP)-conjugated swine anti-rabbit IgG antibody (diluted 1:2000 in blocking buffer; DAKOPATTS A/S) for 3 h. Bound HRP was visualized using enhanced chemiluminescence (ECL) Western-blotting reagent (Amersham Pharmacia Biotech).

Alkaline extraction of membrane proteins

Alkaline extraction of membrane proteins was performed as described [44]. Briefly, 2×10^7 3T3-L1 cells, differentiated for 7 days, were washed twice in PBS and resuspended either in 8 ml of alkaline buffer (0.1 M Na_2CO_3), high salt buffer (0.8 M potassium acetate) or detergent buffer (0.1 % Triton X-100), each containing protease inhibitor cocktail (Boehringer Mannheim), and homogenized with 20 strokes of a Teflon-glass homogenizer (Kontes Glass Co.). Nuclei and unbroken cells were removed by centrifugation for 1000 g for 10 min. Membranes and soluble fractions were separated by centrifugation at 356000 g_{max} for 30 min and the pellets were resuspended in their respective homogenization buffers. Proteins from soluble and membrane fractions were precipitated with 1 vol. of chloroform and 0.25 vol. of methanol, resuspended in 1 % SDS and quantified using the Bio-Rad protein assay according to the manufacturer's instructions. A portion (10 μg) of proteins from each sample was separated by SDS/PAGE and analysed by Western blot.

Subcellular fractionation

All manipulations were carried out at 4 °C. 3T3-L1 cells (1.6×10^8), differentiated for 7 days, were fractionated according to the protocol of Simpson et al. [45] with slight modifications. Briefly, cells were washed twice in PBS resuspended in 8 ml of sucrose holding buffer (SHB; 20 mM Tris/HCl, pH 7.4, 1 mM EDTA, 255 mM sucrose) containing protease inhibitor cocktail (Boehringer Mannheim) and homogenized with 20 strokes in a Teflon-glass homogenizer. Nuclei were pelleted by centrifugation at 1000 g for 10 min. The post-nuclear supernatant was centrifuged at 16000 g_{max} for 20 min. The pellet containing mitochondria, peroxisomes and plasma membrane fractions was resuspended in 5 ml of SHB and layered on to 5 ml of sucrose cushion (1.12 M sucrose, 1 mM EDTA, 20 mM Tris/HCl, pH 7.4) and centrifuged at 101000 g_{max} for 25 min. The mitochondria and peroxisomes were collected as a pellet and resuspended in SHB. The plasma membranes were collected at the interface, resuspended in 10 ml of SHB, centrifuged at 16000 g_{max} for 15 min and resuspended in SHB. The initial 16000 g_{max} supernatant was centrifuged at 48000 g_{max} for 20 min yielding a pellet of high density microsomes (HDM). The supernatant was centrifuged at 212000 g_{max} for 70 min to produce a pellet containing low density microsomes (LDM) and the supernatant containing the cytosol. Proteins from the cytosolic fraction were precipitated with 1 vol. of chloroform and 0.25 vol. of methanol and resuspended in 1 % SDS. Pellets from fractions containing HDM and LDM were resuspended in SHB. Proteins from all fractions were quantified by the Bio-Rad protein assay. A portion (15 μg) of proteins from each sample was separated by SDS/PAGE and analysed by Western blotting using antisera against DD16, G-protein β-subunit (G_β; Santa Cruz), catalase, peroxisome proliferator activated receptor γ (PPARγ; Santa Cruz), glucose transporter isoform 4 (GLUT4) and tryptophanyl tRNA synthetase (WRS).

Biotinylation of cell surface proteins

Biotinylation of cell surface proteins was performed essentially as described [46] with minor modifications. Briefly, 1×10^7 3T3-L1 cells, differentiated for 7 days, were washed twice in PBS, scraped off the tissue-culture dish, centrifuged and resuspended in 1 ml of PBS. Sulphosuccinimidyl-6-biotinamidohexanoate (NHS-LC-biotin, Pierce) was dissolved in DMSO at 20 mg/ml and added to the 3T3-L1 cells at a final concentration of 100 μg/ml. Vials were rotated at room temperature for 30 min and washed three times with 1 ml of PBS. Cells were lysed by adding 1 ml of 50 mM Tris/HCl, pH 7.5, 0.6 % Triton X-100, 5 mM $MgCl_2$ for 30 min on ice, diluted with 500 μl of 50 mM Tris/HCl, pH 7.5, 0.5 % Nonidet P40, 150 mM NaCl, 5 mM EDTA and incubated for 10 min on ice. After centrifugation at 10000 g for 3 min at 4 °C, the supernatant was

transferred to a new tube and 5 μl of rabbit antisera against DD16, G_β or DD16 preimmune serum was added. Vials were rotated for 2 h at 4 °C. Subsequently, 50 μl of 50 % prewashed Protein A–Sepharose (Pharmacia) was added and the vials were rotated for a further 30 min at 4 °C. Protein A–Sepharose beads were washed four times with 50 mM Tris/HCl, pH 7.5, 0.5 % Nonidet P40, 150 mM NaCl, 5 mM EDTA and bound proteins were eluted by boiling in SDS sample buffer (Novex), separated by SDS/PAGE using 4–12 % NuPAGE gels (Novex), and transferred to a nitrocellulose membrane (Kem-En-Tec) using a semi dry blotter (Kem-En-Tec). The membrane was incubated for 2 h in blocking buffer followed by HRP-conjugated streptavidin (diluted 1:20000 in blocking buffer; Kirkegaard & Perry Laboratories) for 3 h. Biotinylated surface proteins were visualized using ECL Western-blotting reagent (Amersham Pharmacia Biotech).

RESULTS

Identification of a novel protein induced during adipocyte differentiation

To identify novel genes that are induced or repressed during differentiation of 3T3-L1 cells, we performed mRNA differential display analysis. Total RNA was prepared from undifferentiated 3T3-L1 cells 2 days post-confluence (referred to as day 0) and cells treated to differentiate for 1 and 7 days with a combination of dexamethasone, insulin and BRL49653. cDNAs derived from these RNAs were then used in mRNA differential display PCR reactions using different arbitrary 5′-primers. A number of ^{32}P-labelled PCR products were revealed in sequencing gels to be differentially regulated during the course of differentiation. One of the PCR products, DD16, was specifically up-regulated after 7 days of differentiation (Figure 1A). The induction of DD16 mRNA was confirmed by Northern-blot analysis using total RNA isolated from 3T3-L1 cells at different time points during differentiation (Figure 1B). Increased expression of a single transcript, with a size of approx. 2.2 kb, was found after 3 days of differentiation. Analysis of the importance of each of the adipogenic compounds revealed that the induction of DD16 mRNA was independent of BRL49653, since treatment of the cells with a combination of dexamethasone and insulin was as potent in inducing DD16 expression as treatment of the cells with a combination of dexamethasone, insulin and BRL49653 (Figure 1B). However, treatment with either dexamethasone or insulin alone was not sufficient to induce the expression of DD16, a finding consistent with the requirement of both dexamethasone and insulin to induce differentiation of 3T3-L1 cells (Figure 1C).

The nucleotide sequence of DD16 cDNA was determined by sequencing and compared with the genes in GenBank®. Identity was found to several expressed sequence tags but no homology to any genes of known function was found.

A full-length cDNA clone was obtained by 5′RACE using a Marathon Ready cDNA library from mouse brain. A single band with a size of 2219 bp was obtained and sequence analysis revealed an open reading frame of 1245 bp encoding a protein with a predicted molecular mass of 46542 Da (Figure 2A). In agreement with this, *in vitro* transcription and translation of DD16 cDNA obtained by 5′RACE generated a protein with a size of approx. 47 kDa (Figure 2B, lane 1). Several cDNA clones of DD16 were also obtained from differentiated 3T3-L1 cells by PCR, which were all identical to the clone obtained from mouse brain (results not shown). Comparison of the nucleotide sequence of DD16 with Genbank® showed high homology with a human gene displaying 89 % identity at the nucleotide level [47]. The accession number for this gene is AB033767. To verify that the start codon of DD16 was consistent with that of the human homologue, we used a deleted mutant, in which nucleotides up to an internal *Not*I site (nucleotide position 102–109) was removed, thus deleting the first methionine. In an *in vitro* transcription and translation reaction, this mutant gave no protein product (Figure 2B, lane 2). Furthermore, there are two in-frame stop codons upstream of nucleotide position 64 (Figure 2A).

To examine the tissue distribution of DD16 mRNA we performed Northern-blot analysis using a multiple tissue Northern blot obtained from Clontech (Figure 3). A high level of expression of a 2.2 kb transcript was found in liver, heart and kidney, whereas a lower expression was found in brain and lung. DD16 mRNA was just detectable in spleen, skeletal muscle and testis. To determine the relative level of DD16 mRNA expression in adipose tissue, we isolated total RNA from mouse adipose tissue and liver and compared the level of DD16 mRNA in these tissues using quantitative PCR. As shown in Table 1, the

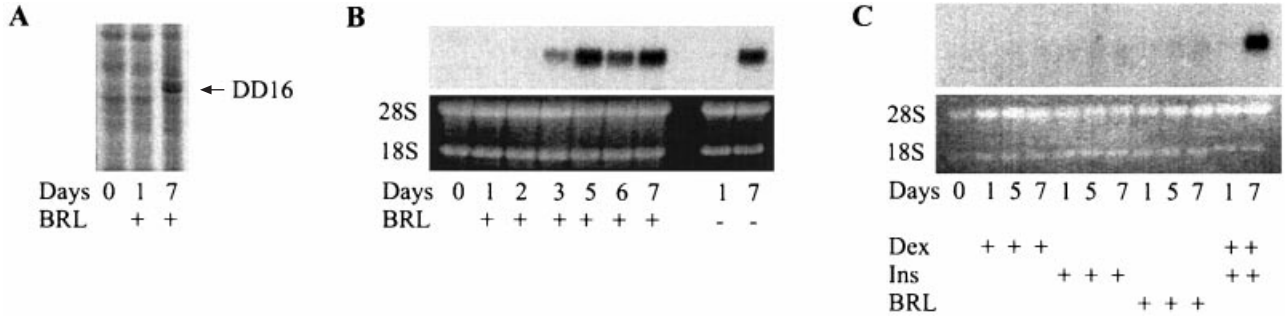

Figure 1 Identification of a novel mRNA induced during differentiation of 3T3-L1 cells

(**A**) mRNA differential display analysis of 3T3-L1 cells induced to differentiate with a combination of dexamethasone and insulin in the presence of BRL49653 (BRL) (+) for the number of days indicated. Day 0 cells are not differentiated and are equivalent to preadipocytes. (**B**) Northern-blot analysis of DD16 expression in 3T3-L1 cells induced to differentiate with a combination of dexamethasone and insulin in the absence (−) or presence of BRL49653 (BRL) (+). (**C**) Northern-blot analysis of DD16 expression in 3T3-L1 cells following treatment with either dexamethasone (Dex), insulin (Ins) and BRL49653 (BRL) alone or in combination. Lower panels in (**B**) and (**C**) show ethidium bromide staining of the membranes with the positions of the 28S and 18S bands indicated.

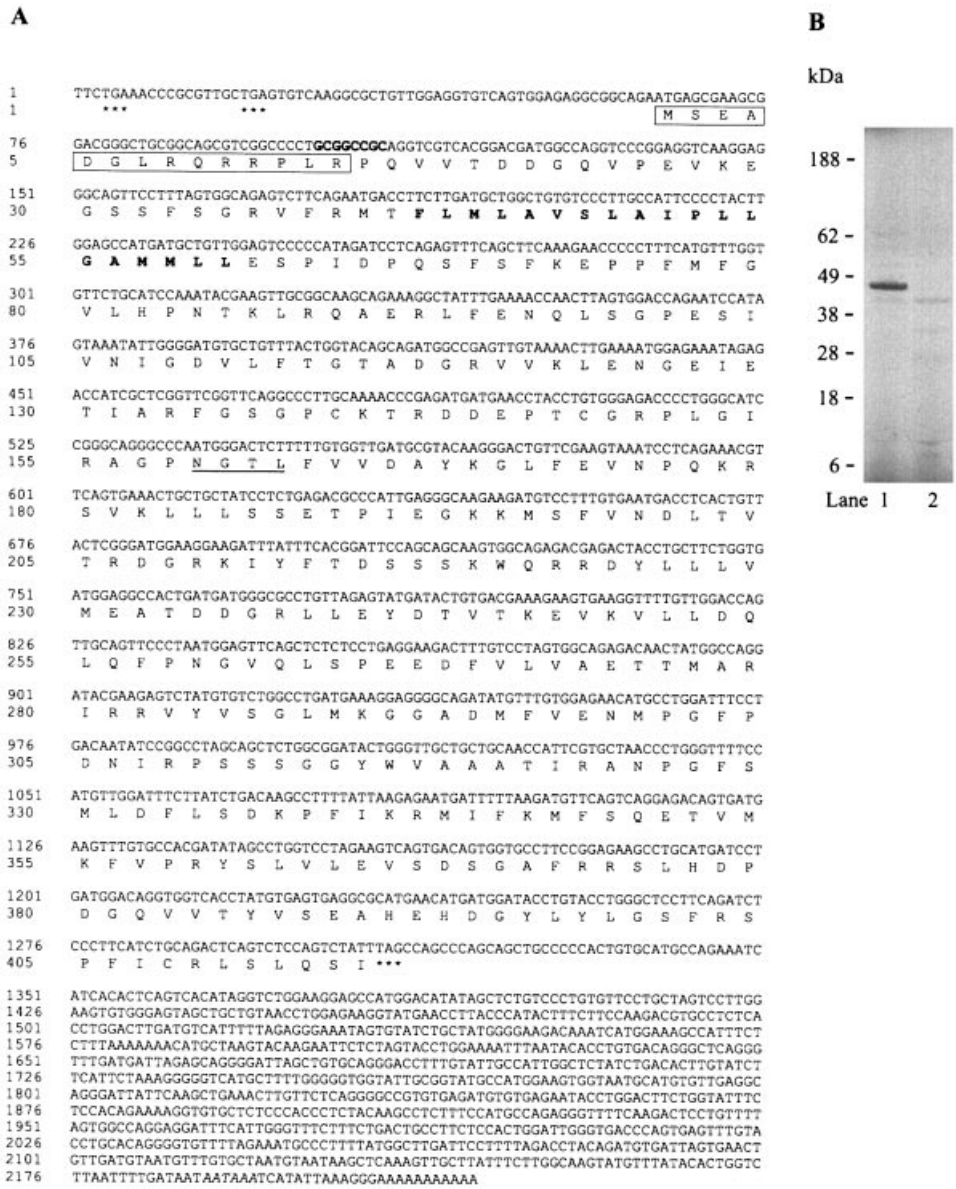

Figure 2 Nucleotide and deduced amino acid sequence of DD16

(**A**) The nucleotide sequence of the cDNA encoding mouse DD16 obtained by 5′-RACE and the amino acid sequence derived from the longest open reading frame are shown. Nucleotide sequence numbers are indicated on the left, and amino acids are numbered in italics on the left starting at the first predicted methionine. In-frame stop codons at the 5′-untranslated region, as well as the in-frame stop codon at the end of the predicted coding region, are denoted by asterisks. The hydrophobic sequence (amino acids 42–60) is indicated in bold and the N-terminal peptide section (amino acids 1–14) selected for antibody production is boxed. A putative polyadenylation signal (nucleotides 2190–2195) is shown in italics. An internal *Not*I site (nucleotides 102–109) is indicated in bold italics and a putative N-glycosylation site (amino acids 159–162) is underlined. (**B**) *In vitro* transcription and translation of DD16 cDNA. The DD16 fragment obtained by 5′-RACE (lane 1) and DD16 fragment from the internal *Not*I site (lane 2), both cloned into a vector downstream of T7 RNA polymerase binding site, were *in vitro* transcribed/translated using T7 RNA polymerase and the TNT *in vitro* translation system (Promega) in the presence of [^{35}S]methionine. Protein products were separated by SDS/PAGE and visualized by autoradiography. Molecular-mass markers (kDa) are shown on the left.

level of DD16 mRNA expression was 4.3-fold higher in adipose tissue compared with the level of expression in liver. Furthermore, we also analysed the level of DD16 mRNA in the adipose tissue and livers of fasted mice; however, no differential expression of DD16 was found (Table 1).

DD16 is a glycosylated protein

Analysis of the DD16 amino acid sequence revealed one long hydrophobic stretch of 19 amino acids (amino acids 42–60) (Figure 2A), which may represent a transmembrane region. Alternatively, the hydrophobic stretch might be a signal peptide. To elucidate the function of the hydrophobic stretch, DD16 was *in vitro* transcribed and translated in the presence of microsomal membranes derived from canine pancreas. These membrane preparations contained both signal peptidase and core glycosylation activities and, as illustrated in Figure 4, increasing amounts of canine microsomal membranes resulted in a reduced mobility of DD16, suggesting that DD16 was glycosylated and not cleaved. In agreement with these findings, a computer-assisted homology

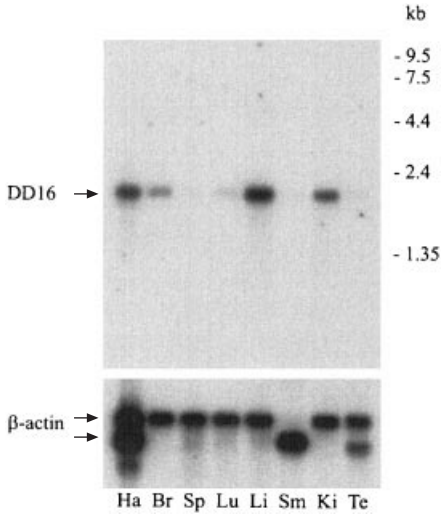

Figure 3 Tissue distribution of DD16 mRNA in adult mice

A Northern blot containing mouse poly(A^+) RNA (2 μg per lane) from the indicated tissues was hybridized with the cDNA fragment of DD16 isolated by mRNA differential display. Hybridization with β-actin was used to demonstrate equal RNA loading in all lanes. Tissues are designated as follows: Ha, heart; Br, brain; Sp, spleen; Lu, lung; Li, liver; Sm, skeletal muscle; Ki, kidney; Te, testis. Marker lengths (kb) are shown on the right.

Table 1 Relative expression of DD16 mRNA in adipose tissue and liver of fed and fasted mice

Data are arbitrary numbers from quantitative PCR using real-time fluorescent detection of DD16 mRNA levels and are presented as means $\pm$ S.E.M. ($n = 3$). *Statistically significant difference ($P \leqslant 0.05$) compared with the corresponding values in adipose tissue.

	Expression level	
Tissue	Fed	Fasted
Adipose tissue	189 $\pm$ 9.1	201 $\pm$ 10
Liver	44.0 $\pm$ 4.9*	46.7 $\pm$ 3.8*

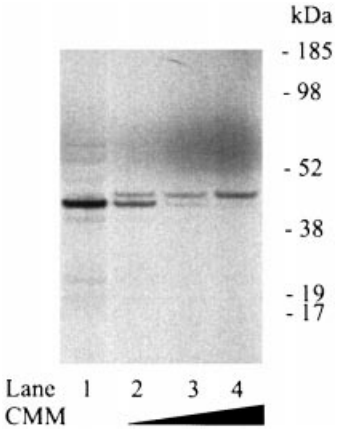

Figure 4 *In vitro* glycosylation of DD16

In vitro transcription/translation of the DD16 cDNA under standard conditions (lane 1) and with increasing amounts of canine pancreatic microsomal membranes (CMM; 0.3, 0.9 μl and 1.8 μl). Molecular-mass markers (kDa) are indicated on the right.

search revealed the presence of a putative N-glycosylation site at amino acid position 159–162 (Figure 2A). Furthermore, the transmembrane hidden Markov model ('TMHMM') [48] predicted that the N-terminal hydrophobic stretch was a transmembrane helix (results not shown).

Generation of anti-DD16 antiserum

To facilitate the further studies of DD16 we generated a rabbit polyclonal anti-peptide serum directed against amino acid residues 1–14 of DD16. The antiserum was initially tested for its ability to immunoprecipitate *in vitro* transcribed and translated DD16 protein. As illustrated in Figure 5(A), no product was precipitated with preimmune serum, whereas anti-DD16 serum resulted in the precipitation of *in vitro* transcribed and translated DD16. Subsequently, we tested the ability of the antiserum to recognize DD16 in differentiated 3T3-L1 cells by Western-blot analysis. In accordance with the Northern-blot analysis, no DD16 could be detected in 3T3-L1 preadipocytes, whereas a band with the predicted molecular mass was detected in 3T3-L1 adipocytes (Figure 5B). Taken together with the obervation that the DD16 signal was inhibited by incubation with the immunizing peptide (Figure 5C), these results demonstrate that the antiserum specifically recognized the adipocyte DD16 protein.

DD16 is an integral membrane protein

The results presented in Figure 4 suggest that DD16 is a glycosylated membrane-associated protein. To verify this we separated soluble and membrane-bound proteins from differentiated 3T3-L1 cells by extraction in alkaline, high salt or detergent-containing buffers, and subjected both fractions to Western-blot analysis. DD16 remained associated with the membrane fractions following treatment with the alkaline- or high salt-buffers, whereas the presence of detergent resulted in DD16 being located in the soluble fraction (Figure 6). In summary, these results therefore suggest that DD16 is an integral membrane protein.

DD16 is associated with the adipocyte plasma membrane

The presence of DD16 in the membrane fraction preparation prompted a detailed analysis of the subcellular distribution of DD16. This was done by subcellular fractionation of differentiated 3T3-L1 cells using differential density centrifugation to yield membrane fractions enriched for nuclei, peroxisomes/mitochondria, plasma membrane, HDM, LDM and cytosol. Proteins from each fraction were separated by SDS/PAGE and analysed by Western-blot analysis using polyclonal anti-DD16 serum (Figure 7A). High levels of DD16 were detected in the plasma membrane and HDM fractions with slightly lower amounts found in peroxisomes/mitochondria. However, no DD16 was detected in nuclear, LDM or cytosolic fractions. To verify the identity of these fractions, the distribution of proteins known to reside within specific intracellular compartments were analysed by Western-blot analysis. G_{β}, known to be associated with the plasma membrane, was detected primarily in the plasma membrane fraction and also at slightly lower levels in the HDM and peroxisomal/mitochondrial fractions. The peroxisomal enzyme α-catalase was enriched in the peroxisomal/mitochondrial fraction and relatively depleted from the plasma membrane and HDM fractions. PPARγ, a member of the nuclear hormone

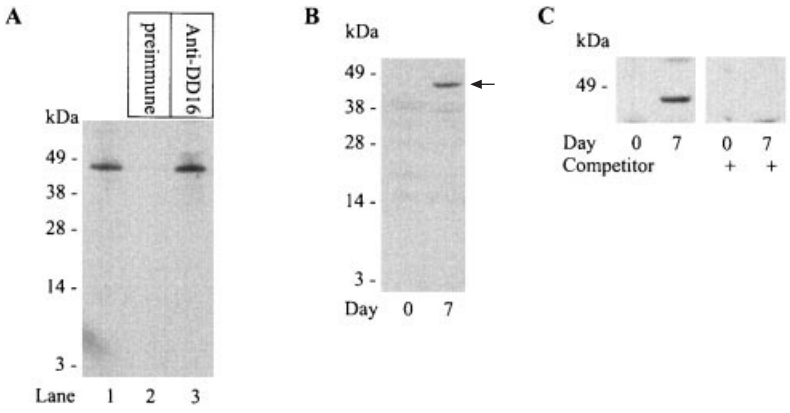

Figure 5 Characterization of anti-DD16 serum

(**A**) The DD16 cDNA was *in vitro* transcribed and translated using the TNT *in vitro* translation system in the presence of [^{35}S]methionine (lane 1) and immunoprecipitated either with rabbit preimmune serum (lane 2) or rabbit polyclonal anti-peptide serum raised against the amino terminus of DD16 (lane 3). Precipitated proteins were separated by SDS/PAGE and visualized by autoradiography. (**B**) Proteins from 3T3-L1 preadipocytes (day 0) and from 3T3-L1 cells differentiated for 7 days were separated by SDS/PAGE and immunoblotted using rabbit anti-DD16 serum. The arrow indicates DD16. (**C**) Proteins were prepared and analysed as described in (**B**) and, as a control, the immunizing amino terminus peptide (residues 1–14) was included in the indicated lanes at a concentration of 50 μg/ml. Molecular-mass markers (kDa) are indicated on the left.

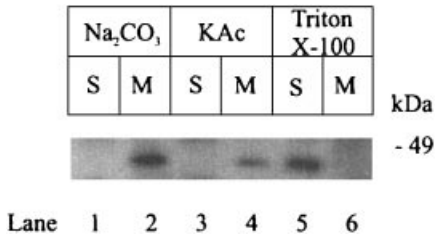

Figure 6 DD16 is an integral membrane protein

3T3-L1 cells differentiated for 7 days were homogenized in 0.1 M Na_2CO_3 (lanes 1 and 2), 0.8 M potassium acetate (KAc; lanes 3 and 4), and 1% Triton X-100 (lanes 5 and 6). Soluble (S) and membrane (M) fractions were separated by centrifugation, proteins were precipitated and 10 μg of protein from each fraction was separated by SDS/PAGE and immunoblotted using rabbit anti-DD16 serum.

receptor superfamily and known to play an important role in adipocyte differentiation, was only detected in the nuclear fraction. GLUT4, which has previously been shown to be associated with the HDM and LDM and, after insulin treatment, also associated with the plasma membrane [49], was highly enriched in the plasma membrane and HDM fractions. Finally, WRS, known to be localized in the cytoplasm and nucleus [50], was detected in these fractions only. Taken together these results suggest that DD16 is a novel plasma membrane-associated protein. Its appearance in the HDM fraction may represent newly synthesized proteins that have not yet reached the plasma membrane. The low, but detectable, expression of DD16 in the peroxisomal/mitochondrial fraction may be real, but may also result simply from contamination of this fraction with plasma membrane and/or HDM fractions.

To validate if DD16 is an integral plasma membrane protein in differentiated 3T3-L1 cells, biotinylation of cell surface proteins followed by lysis of the cells and immunoprecipitation of DD16 using anti-DD16 serum was performed. Biotinylation of DD16 was visualized by Western-blot analysis of immunoprecipitated proteins using HRP-coupled streptavidin and ECL. Immunoprecipitation using anti-DD16 serum resulted in a band corresponding to the size of DD16 (Figure 7B, lane 2). No bands were detected using preimmune serum (Figure 7B, lane 1) or antibodies raised against G_β, which, as described above, is an intracellular plasma membrane-associated protein (Figure 7B, lane 3). These biotinylation studies of surface proteins are in agreement with our subcellular fractionation data and strongly suggest that DD16 is an integral plasma membrane protein. We have therefore suggested this protein be named Adipocyte Plasma Membrane-Associated Protein (APMAP).

DD16 is a novel marker of adipocyte differentiation

Although the differentiation of 3T3-L1 preadipocytes mimics the process of adipocyte differentiation *in vivo*, this model system lacks the endo- and paracrine-interactions between cell types found *in vivo*. We therefore analysed the expression of APMAP during the course of differentiation of primary cultures of rat preadipocytes. Primary epididymal preadipocytes were isolated from Sprague–Dawley rats and cultured in serum-free medium supplemented with insulin, T_3 and BRL49653. Marked differentiation of the cells occurred during the first 5 days with induction of adipocyte markers such as aP2 and LPL. Determination of the expression levels of aP2, LPL and APMAP in three independent differentiation experiments showed that aP2 was increased by at least 86-fold after 5 days of differentiation, while the levels of LPL and APMAP were elevated at least 18- and 13-fold, respectively.

Previous reports have shown that addition of TNFα to differentiated adipocytes causes a down-regulation of adipocyte specific genes such as PPARγ and FSP27 [51]. Therefore to determine whether TNFα had an effect on the expression pattern of APMAP, differentiated 3T3-L1 cells were treated with TNFα and analysed after various time intervals. As expected, TNFα reduced the PPARγ mRNA level between 1 and 6 h after addition to the cells and, interestingly, a clear reduction in the APMAP mRNA level was detected over the same time interval. In contrast, TNFα had no effect on the expression level of 36B4 (Figure 8). Taken together with the increased expression of

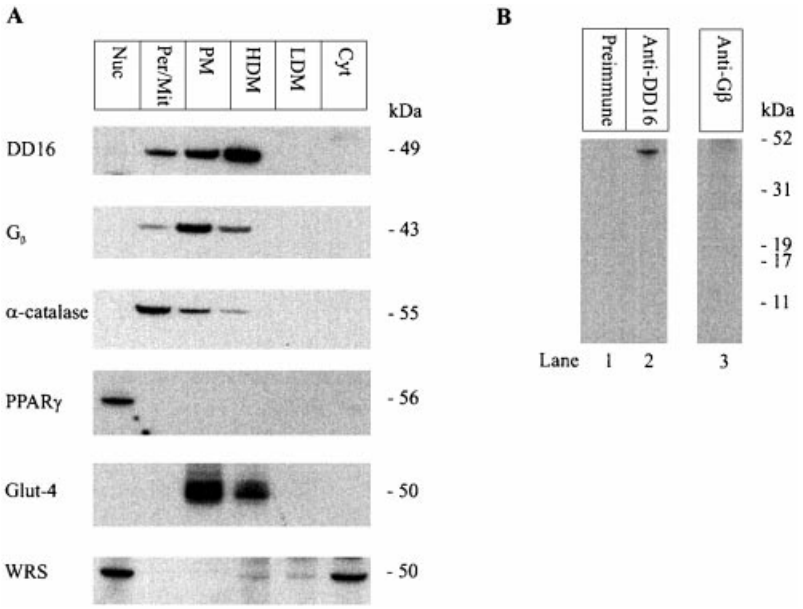

Figure 7 Intracellular localization of DD16 in adipocytes

(**A**) 3T3-L1 cells differentiated for 7 days were disrupted by homogenization and fractionated by differential density centrifugation. A portion (15 μg) of protein from each fraction was separated by SDS/PAGE and immunoblotted using rabbit anti-DD16 serum. Fractions are designated nucleus (Nuc), peroxisomes/mitochondria (Per/Mit), plasma membrane (PM), HDM, LDM and cytosol (Cyt). Antisera against marker proteins (G_β, α-catalase, PPARγ, GLUT4 and WRS) were used to assess the enrichment of the various fractions. (**B**) Differentiated 3T3-L1 cells were labelled with 100 μg/ml of NHS-LC-biotin for 30 min. Cells were lysed and immunoprecipitation was carried out using DD16 preimmune serum (lane 1), anti-DD16 serum (lane 2) or anti-G_β serum (lane 3). Immunoprecipitates were separated by SDS/PAGE and transferred on to nitrocellulose filter. Biotinylated proteins were visualized by incubation with HRP-coupled streptavidin. Molecular-mass markers (kDa) are indicated on the right.

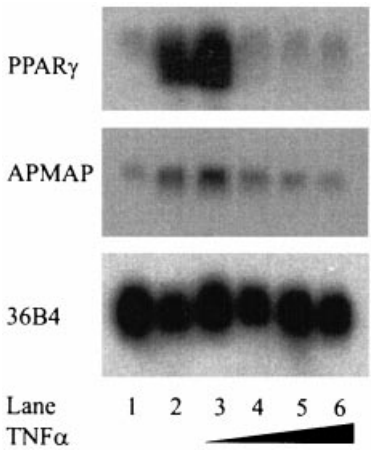

Figure 8 Time course reversal of APMAP expression by TNFα

3T3-L1 cells, 2 days post-confluence (lane 1), were differentiated for 7 days with dexamethasone and insulin (lane 2) after which they were treated with 25 ng/ml TNFα for 1 h (lane 3), 6 h (lane 4), 24 h (lane 5) and 48 h (lane 6). Total RNA was isolated and 5 μg was used for Northern-blot analysis to detect expression levels of PPARγ, APMAP and 36B4.

APMAP during the course of adipocyte differentiation of primary rat preadipocytes, our results therefore strongly indicate that APMAP is characteristic of the mature adipocyte and may serve as a novel marker of adipocyte differentiation.

DISCUSSION

White adipose tissue is the major organ for storage of triacylglycerols in mammals. By expression of a variety of proteins involved in lipid metabolism, as well as several secreted molecules, this tissue plays a key role in the control of energy balance. Imbalance in the abundance of white adipose tissue increases the risk of several pathological disorders, including coronary heart disease, cancer, hypertension and type 2 diabetes, and the dramatic increase in excess of white adipose tissue, particularly in industrialized countries, is a major public health problem. The mechanism by which obesity causes the incidence of these disorders is at present unclear. However, a general hypothesis is that the regulation of white adipose tissue differentiation and the modulation of expression of specific genes in the mature adipose cells may influence the propensity for disorders.

To identify novel genes that might be involved in these processes, we have identified a set of mRNAs in 3T3-L1 cells exhibiting altered expression during the process of differentiation in these cells. Among these mRNAs, we have identified an mRNA encoding a novel protein, which is abundantly expressed in differentiated 3T3-L1 cells, but absent in non-adipose cells. The induction of this novel mRNA was also observed during the course of differentiation of primary rat preadipocyte cultures, strongly indicating that the increased expression observed in 3T3-L1 adipocytes is not an artefact stemming from this immortalized cell line.

Several lines of evidence suggest that the protein encoded by the identified mRNA is an integral plasma membrane-associated protein, and we therefore suggest this protein be named APMAP (Adipocyte Plasma Membrane-Associated Protein). First,

APMAP contains a hydrophobic stretch of 19 amino acids, which is sufficient for the formation of an α-helix that spans a phospholipid bilayer. Secondly, APMAP associates with the membrane fractions of 3T3-L1 adipocytes following treatment with alkaline- and high salt-buffers. Thirdly, APMAP can be found in the plasma membrane fraction of 3T3-L1 adipocytes after subcellular fractionation. Finally, biotinylation of surface proteins in 3T3-L1 adipocytes with a compound known to be unable to penetrate the cell membrane, followed by lysis of the cells and immunoprecipitation of APMAP, shows biotinylation of APMAP.

In addition, APMAP is glycosylated *in vitro*, which is consistent with a putative N-glycosylation site found by the use of a computer-assisted sequence homology search. The putative N-glycosylation site is found at amino acid position 159–162, suggesting that the C-terminal part of APMAP, corresponding to amino acid 61–415, composes the extracellular site of the protein. Since protein glycosylation occurs in the lumen of endoplasmic reticulum (ER), the C-terminal portion of APMAP is therefore expected to be localized in the lumen of the ER during the process of glycosylation. The orientation of peptides in the ER membrane, as well as other membranes, depends on the distribution of nearby charged amino acids [48,52]. In peptides in which the C-terminal part is localized in the lumen of ER, more positively charged amino acids are found immediately preceding the hydrophobic stretch compared with the amino acids found immediately after the hydrophobic stretch. In agreement with this, two arginines are found in APMAP just before the hydrophobic stretch.

Taken together, our results strongly suggest that APMAP is a novel glycosylated integral plasma membrane-associated protein containing a large extracellular domain composed of 355 amino acids and a rather small intracellular domain of 41 amino acids, in which case APMAP is a class II membrane protein [52]. The biological function of APMAP is, however, presently unknown. A computer-assisted sequence homology search revealed the presence of putative casein kinase 2 and protein kinase C phosphorylation sites at the putative intracellular N-terminal part of APMAP. However, several of these phosphorylation sites are scattered throughout the protein and the significance of these sites is speculative. The existence of the large extracellular domain makes it tempting to speculate that this domain of APMAP functions as a binding domain mediating interaction with a small peptide or another signalling molecule, or alternatively mediates contact with neighbouring cells or the extracellular matrix. In agreement with this hypothesis, APMAP has a low, but significant, similarity (31% identity and 50% homology between amino acids) to hemomucin, a *Drosophila* surface mucin, probably involved in the induction of anti-bacterial effector molecules after binding to lectins [53]. However, no homology was found to the mucin domain, indicating that APMAP is not involved in lectin interactions. The functional relevance of homology between hemomucin and APMAP therefore remains to be determined. Studies are currently underway in our laboratory to attempt to delineate the function of APMAP.

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