Purification of the major substrate for palmitoylation in rat adipocytes: N-terminal homology with CD36 and evidence for cell surface acylation

Albert Jochen[1] **and Judith Hays**

Department of Medicine, Medical College of Wisconsin, Zablocki Veterans Administration Hospital, Milwaukee, WI 53295

Abstract We previously reported that incubating rat adipocytes with [^{3}H]palmitate predominantly labeled an 85-kDa protein. The labeling was more intensive in the presence of insulin and had characteristics consistent with covalent fatty acylation (Jochen et al. 1991. *Biochem. Biophys. Res. Commun.* **177:** 797–801). In order to determine the significance of this finding we purified the 85-kDa protein, determined its N-terminal sequence, and further characterized its interactions with long-chain fatty acids. The [^{3}H]palmitate-labeled 85-kDa protein was purified from rat adipocyte membranes using the following sequence of procedures: *i)* affinity chromatography with wheat germ agglutinin-agarose, *ii)* ion exchange chromatography with DEAE-Sepharose, and *iii)* SDS-polyacrylamide gel electrophoresis. The resulting labeled protein was sequenced through 30 amino acid residues. With the exception of one conserved substitution, the sequence was identical to CD36 (platelet membrane glycoprotein IV). Further characterization of the 85-kDa protein revealed it was heavily N-glycosylated and possessed a cell surface domain. Labeling of the 85-kDa protein with palmitate was compared in control cells, insulin-treated cells, and cells whose energy was depleted with 2,4-dinitrophenol. Insulin and energy depletion increased labeling approximately 3-fold and 12-fold, respectively. Labeling performed in the presence of energy depletion possessed all of the characteristics of covalent protein acylation. In addition, there was a close association between the ability of energy depletion to increase labeling of the 85-kDa protein and its ability to inhibit depletion of [^{3}H]palmitate from the extracellular incubation media. These results suggest that the major substrate for fatty acylation in adipocytes is a cell surface membrane protein related to CD36 and that this acylation has the unusual properties of being independent of intracellular metabolic energy and of occurring on an exofacial epitope of the protein.—**Jochen, A., and J. Hays.** Purification of the major substrate for palmitoylation in rat adipocytes: N-terminal homology with CD36 and evidence for cell surface acylation. *J. Lipid Res.* 1993. **34:** 1783–1792.

Supplementary key words 85-kDa protein • platelet membrane glycoprotein IV • insulin • energy depletion

Acylation of proteins by long-chain fatty acids occurs ubiquitously in eucaryotic cells and is important to the function of some proteins (reviewed in references 1–4). Several types of covalent fatty acid linkage to proteins have been described. One type involves thioester or oxyester linkages to internal cysteine, serine, or threonine residues and utilizes a variety of fatty acids including palmitate, stearate, and oleate. These linkages are catalyzed by specific protein fatty acyltransferases, are readily reversed enzymatically by deacylases (5), and are generally formed post-translationally in mature proteins. A second type of acylation involves the linkage of myristate to amino-terminal glycine residues. This process, known as myristoylation, occurs co-translationally and is important in targeting newly synthesized proteins to their correct cellular destinations by stabilizing their interactions with membranes (6). Fatty acids are also incorporated into proteins via more complex linkages such as those found in proteins modified by glycan-phosphoinosotides (7) and those occurring on N-terminal cysteine residues of certain bacterial membrane proteins (8). While the functional significances of these various types of protein acylation are not entirely defined, several lines of evidence suggest that the type of acylation involving palmitoylation via thioester linkages has particular relevance to membrane trafficking (9–15).

Because protein acylation by long-chain fatty acids is relevant to membrane trafficking, and because several hormonal effects in adipocytes depend upon membrane trafficking (16–18), it would be of interest to identify the major adipocyte membrane proteins that are substrates for palmitoylation. In a preliminary study we found that incubating intact rat adipocytes with [^{3}H]palmitate predominantly labeled an 80- to 85-kDa protein. The labeling had characteristics suggestive of covalent fatty

[1]To whom correspondence should be addressed at: Medical College of Wisconsin, Division of Endocrinology and Metabolism, 9200 West Wisconsin Avenue, Milwaukee, WI 53226.

acylation, including sensitivity of the linkage to neutral hydroxylamine and recovery of released label as unaltered [^{3}H]palmitate (19). Furthermore, the intensity of labeling was increased in the presence of insulin. Whether this was a direct effect of insulin stimulating a protein fatty acyltransferase or an indirect effect on fatty acid pools was undetermined. In this study, we further characterized the interaction of the 80- to 85-kDa protein with fatty acids and determined its N-terminal sequence. We report here that the protein is related to leukocyte differentiation antigen CD36 and its acylation has several novel and unusual features suggesting that long-chain fatty acids acylate this protein on a cell surface domain largely independent of intracellular metabolic energy.

EXPERIMENTAL PROCEDURES

Isolation of rat adipocytes

Isolated adipocytes were prepared by collagenase digestion (20) of epididymal fat pads obtained from male Sprague-Dawley rats weighing 200–350 g, fed ad libitum. The cells were filtered through a 250-μm nylon mesh, washed four times, and suspended in phosphate-buffered saline (PBS), pH 7.5, containing 1% defatted bovine serum albumin (Sigma Co., St. Louis, MO). To reduce basal lipolysis all incubations contained 100 nM phenylisopropyladenosine (21).

Purification of labeled 85-kDa protein from adipocyte membranes

Isolated adipocytes from four to five rats were incubated at a lipocrit of 25% in 20 ml PBS, pH 7.5, containing 100 nM phenylisopropyl adenosine, 1% defatted bovine serum albumin, and 30 ng/ml insulin for 20 min at 37°C. [^{3}H]palmitate (NEN DuPont, Wilmington, DE) was added (200 μCi/ml) and after further incubation for 60 min at 37°C the adipocytes were washed three times with PBS and then homogenized in three volumes of 50 mM sucrose, 1 mM EDTA, 20 mM Tris, pH 7.4. The homogenate was centrifuged at 3000 *g* for 5 min to remove cellular debris and then centrifuged at 100,000 *g* for 60 min to produce crude membranes. The membranes were solubilized in 1% Triton, 20 mM Tris, 2 mM β-mercaptoethanol, 10 mM EDTA, pH 7.6. The solubilized membrane proteins were precipitated with six volumes of ether–ethanol 1:1, washed twice with ether–ethanol 1:1, and then repeatedly washed with chloroform–methanol 2:1 until no further radioactivity could be removed. The proteins were resolubilized, clarified by centrifugation, and applied to a 1.5 ml wheat germ agglutinin-agarose column. The column was washed with 100 ml of 0.2% Triton, 20 mM Tris, pH 7.6, after which affinity-bound proteins were eluted with 0.3 M N-acetyl glucosamine, 0.2% Triton, 20 mM Tris, pH 7.6, and collected in 1-ml fractions. The eluted proteins were dialyzed overnight against 10 mM ammonium bicarbonate, pH 7, and then dried. The proteins were resolubilized in 20 mM Tris, 0.2% Triton, pH 8, and re-dialyzed against 20 mM Tris, 0.2% Triton, pH 8. Membrane glycoproteins pooled from 30 rats (ca 2 mg) were then applied to a 1.5 × 5 cm DEAE-Sepharose column. Bound proteins were eluted with an increasing salt gradient. The starting buffer was 20 mM Tris, 0.2% Triton, pH 8, and the final buffer was 20 mM Tris, 0.2% Triton, 0.5 M NaCl, pH 8. One-ml fractions were collected and assayed for protein content and radioactivity. Protein assays were performed with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA). Gel electrophoresis and autoradiography of purified proteins were performed as described below.

Sequencing of N-terminus of labeled 85-kDa protein

Approximately 20 μg of purified 85-kDa protein was subjected to SDS-polyacrylamide gel electrophoresis and then electroblotted onto a polyvinylidene difluoride membrane. The blotted protein was subjected to automated Edman degradation. The resulting sequence was compared to sequences of known proteins using the Sequence Analysis Software Package (Genetics Computer Group).

Endoglycosidase F digestion of labeled 85-kDa protein

Purified, ^{3}H-labeled 85-kDa protein (2 μg) was incubated for 8 h at 37°C in 150 μl of PBS, pH 7.4, containing 0.2% Triton, 2 mM β-mercaptoethanol, and 1 unit endoglycosidase F (Sigma Co., St. Louis, MO). Control samples were similarly incubated in the absence of endoglycosidase F. The incubation was terminated by the addition of 200 μl Laemmli's buffer, followed by heating at 100°C for 2 min.

Surface labeling of adipocyte proteins with ^{125}iodine

Isolated adipocytes from four rats were incubated in 20 ml PBS, pH 7.5, with 20 Iodo-Beads (Pierce, Rockford, IL) and 3 mCi carrier-free sodium iodide (NEN DuPont, Wilmington, DE) for 10 min at 8°C (22). Iodo-Beads contain the iodinating agent 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril covalently immobilized to polystyrene beads. The cells were washed and homogenized. Crude membranes were prepared and iodinated 85-kDa protein was purified by lectin affinity chromatography and ion exchange chromatography as described above.

Labeling of adipocyte proteins with [^{3}H]palmitate

To generate labeled proteins for a single experimental point or gel electrophoresis lane (see Figs. 7–11, 13, 14), isolated rat adipocytes (~1,000,000 cells) were incubated, in a 17 × 100 mm polypropylene tube, at 37°C in 2 ml PBS, pH 7.5, containing 100 nM phenylisopropyladenosine and 1% defatted bovine serum albumin. Cells were pre-incubated with insulin and/or energy depletors for 20

min prior to the addition of 400 μCi of [^{3}H]palmitate (NEN DuPont, Wilmington, DE) dissolved in ethanol. The [^{3}H]palmitate stock solution was partially concentrated prior to its addition, producing an ethanol concentration in the incubation of 1% or less. After further incubation for the times indicated, the cells were washed four times with ice-cold PBS and then solubilized with two volumes 1% SDS, 10 mM EDTA, 1 mM PMSF, 20 mM Tris, 2 mM β-mercaptoethanol, pH 7.6. The solubilized cells were transferred to a 15-ml glass Corex tube and the solubilized proteins were then precipitated with six volumes of ice-cold ether–ethanol 1:1. Except where indicated, the precipitate was washed twice with ether–ethanol 1:1 and twice with chloroform–methanol 2:1. The delipidated, precipitated proteins were resolubilized with Laemmli's buffer, boiled for 2 min, and analyzed under reducing conditions by SDS-polyacrylamide gel electrophoresis using 10% acrylamide gels (23). The gels were stained with Coomassie Brilliant Blue, destained, soaked in Enhance (DuPont Co., Wilmington, DE), dried, and autoradiographed at −70°C using X-Omat AR film (Eastman Kodak Co., Rochester, NY) and Cronex Lightning Plus intensifying screens (DuPont Co., Wilmington, DE). The molecular weight markers used were phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (31 kDa).

Quantitation of ^{3}H-labeled 85-kDa protein in SDS-polyacrylamide gels

Areas of dried gels containing the labeled 85-kDa protein were identified by autoradiography, excised, and minced. The fragments were dissolved by heating in 30% hydrogen peroxide for 30 min at 100°C, transferred to scintillation vials, and counted. Areas from the gel containing no labeled protein were also processed and then subtracted as background counts.

Assay of fatty acids in media

The concentration of nonesterified fatty acids in the medium was assayed in chloroform extracts by a modification of the copper sulfate method (24).

RESULTS

The fatty acylated 85-kDa protein was purified from adipocyte membranes by lectin affinity chromatography followed by ion exchange chromatography. **Fig. 1** shows enrichment of the acylated protein by wheat germ agglutinin-agarose chromatography of solubilized, delipidated membrane proteins. Only a small amount of the labeled protein was not bound by the affinity column, rather most labeled protein was bound by the column and specifically eluted by N-acetyl-glucosamine.

Fig. 2 shows further purification of the 85-kDa protein from adipocyte membrane glycoproteins by ion exchange chromatography. Radioactivity eluted in two peaks. The first peak corresponded to the salt volume of the column and, when analyzed by gel electrophoresis, no protein bands were seen and the radioactivity migrated at the front of the gel (data not shown). Thus, material that had reacted with the Bio-Rad protein assay to form this peak was nonproteinaceous, probably representing Triton X-100 concentrated in the sample during the purification steps.

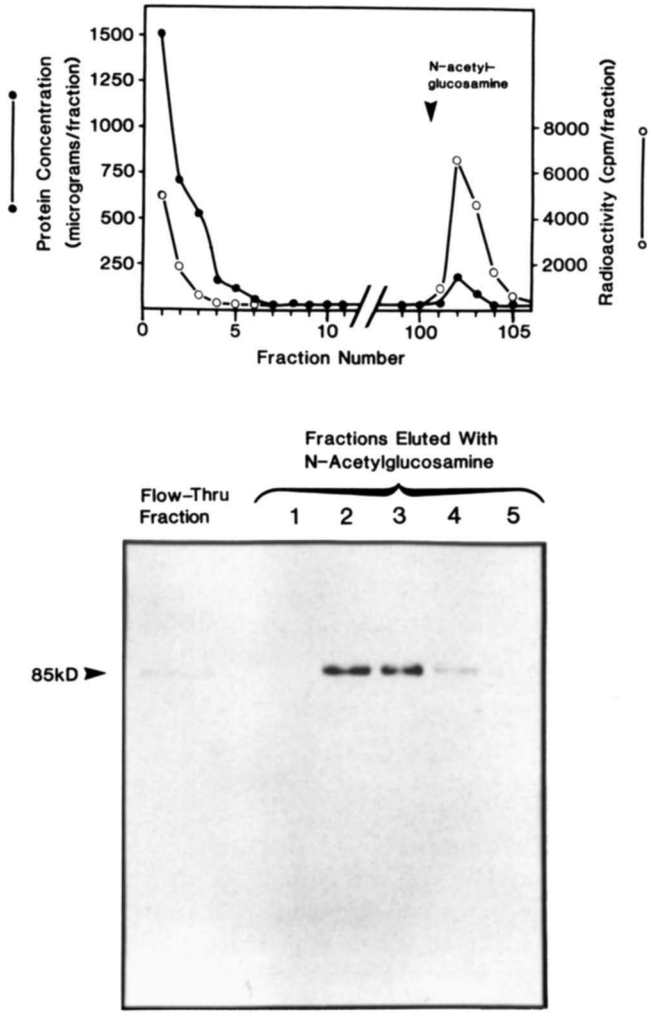

Fig. 1. Enrichment of [^{3}H]palmitate-labeled protein by lectin affinity chromatography. Approximately 3 mg solubilized, labeled membrane proteins (from four rats) was applied to a 1.5-ml wheat germ agglutinin-agarose column. The column was washed, after which affinity-bound glycoproteins were eluted with N-acetyl glucosamine. Top panel: protein concentration and radioactivity in the flow-through, wash, and elution fractions. Bottom panel: SDS-polyacrylamide gel electrophoresis and autoradiography of the initial 3 ml of flow-through and the first five elution fractions.

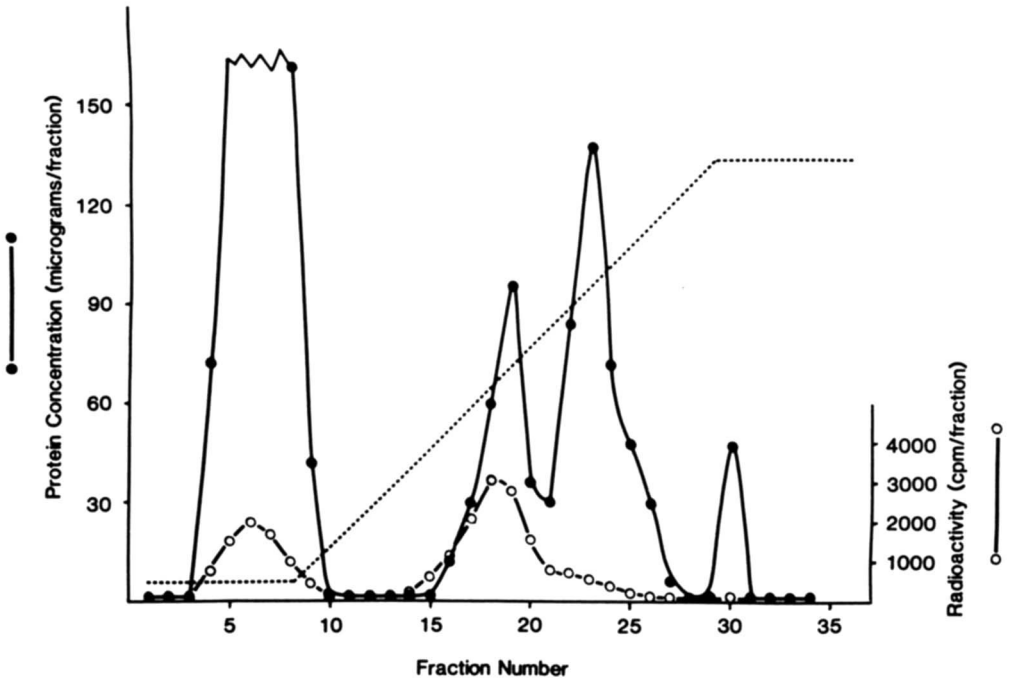

Fig. 2. Ion exchange chromatography of [^{3}H]palmitate-labeled membrane glycoproteins. Approximately 2 mg of labeled membrane glycoproteins (from 30 rats) was applied to a DEAE-Sepharose column and then eluted with an increasing salt gradient (dashed line). Shown are the protein content and radioactivity of each fraction.

Fig. 3 shows total membrane glycoproteins and the second radioactive peak of the ion exchange column analyzed by SDS-polyacrylamide gel electrophoresis. The Coomassie Blue stain (panel A) revealed two dominant membrane glycoproteins of molecular weights 95 kDa and 85 kDa. Radioactivity appearing in the second DEAE-Sepharose peak corresponded to a major protein peak and consisted of highly purified 85-kDa protein (panel A). The corresponding autoradiograph (panel B) demonstrated that the purified 85-kDa protein stained by Coomassie Blue was the dominant radioactive protein. When the labeled 85-kDa protein was analyzed under nonreducing conditions, rather than reducing conditions, no difference in molecular weight was seen (data not shown). Therefore, the protein consists of a single subunit.

The N-terminus of the purified 85-kDa protein was successfully sequenced through 30 amino acids, compared to sequences of known proteins, and found to be highly homologous with leukocyte differentiation antigen CD36 (25). The N-terminal sequences of the fatty acylated 85-kDa protein and CD36 are compared in **Fig. 4.** All amino acids were identical with the exception of residue 10 in which there was a conserved substitution of threonine for alanine. Residues 2 and 6 were not identified in the 85-kDa protein and therefore likely represent cysteines which would be in agreement with the known sequence of CD36.

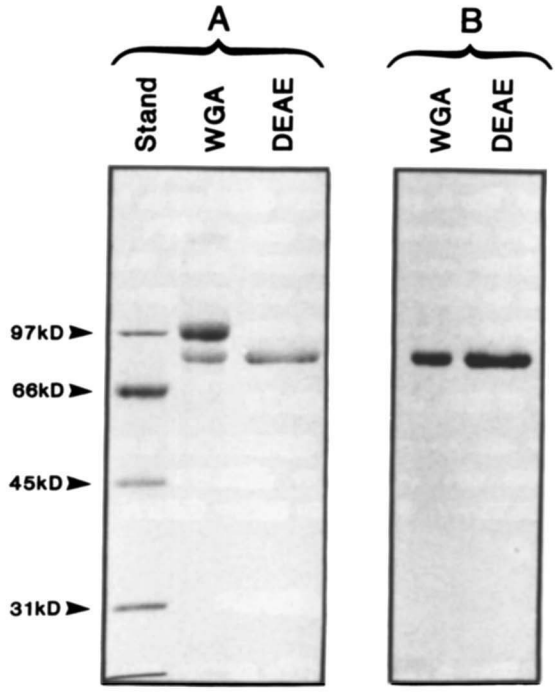

Fig. 3. Coomassie Blue stains and autoradiograms of purified protein fractions analyzed by SDS-polyacrylamide gel electrophoresis. Panel A (Coomassie Blue stain): Stand, standards; WGA, membrane glycoproteins purified by wheat germ agglutinin–agarose chromatography; DEAE, second radioactive peak eluted from DEAE-Sepharose column. Panel B: the corresponding autoradiogram is shown for wheat germ agglutinin–agarose-purified proteins (WGA) and for the second radioactive peak eluted from the DEAE-Sepharose column (DEAE).

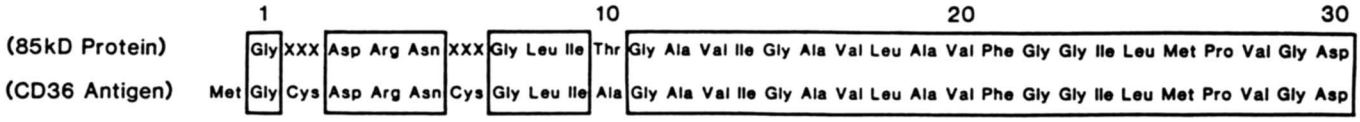

Fig. 4. Comparison of the N-terminal amino acid sequences of the rat adipocyte fatty acylated 85-kDa protein and of human CD36. Regions of homology are boxed.

Fig. 5 demonstrates that the 85-kDa protein is heavily N-glycosylated. Treatment with endoglycosidase F decreased the apparent molecular weight from 85 kDa to 70 kDa. The susceptibility of the 85-kDa protein to cell surface iodination was examined next. Analysis of total membrane glycoproteins revealed two major iodinated cell surface species (**Fig. 6,** lane 1), corresponding to the 95-kDa and 85-kDa proteins visualized by Coomassie Blue staining (Fig. 3, panel A). The iodinated 85-kDa component was further purified by ion exchange chromatography (Fig. 6, lane 2). It eluted in the same position as the fatty acylated 85-kDa protein. Therefore, the 85-kDa protein possesses an extracellular domain.

Fig. 7 compares the effects of insulin and energy depletors on [^{3}H]palmitate labeling of the 85-kDa protein. The concentration of 2,4-dinitrophenol used in this study (1 mM) is large and reduces adipocyte ATP levels by approximately 90% (26). Insulin increased labeling 3-fold while energy depletion increased labeling approximately 12-fold. Sodium fluoride and 2,4-dinitrophenol produced similar levels of palmitoylation. Effects of insulin and energy depletors were nonadditive.

The chemical nature of labeling produced by energy depletors was examined in Fig. 8 through 11. The label was readily removed by neutral hydroxylamine (**Fig. 8**). Sensitivity to hydroxylamine is typical of covalent linkage via thioester or oxyester bonds (1–4). To demonstrate that labeling was an active enzymatic process, effects of low temperature, N-ethylmaleimide, and extracellular trypsin were examined (**Fig. 9** and **Fig. 10**). All of these conditions effectively inhibited labeling of the 85-kDa protein. The trypsin studies were extended by testing sensitivity of the 85-kDa protein to digestion after, rather than before, labeling with [^{3}H]palmitate was complete. Under these conditions, trypsin had no effect on either the 85-kDa protein or its labeling (data not shown). Labeling was also blocked by cerulenin (**Fig. 11**), a fungal antibiotic reported to inhibit protein fatty acylation (14, 15, 27, 28).

To further demonstrate that the interaction between [^{3}H]palmitate and the 85-kDa protein induced by energy depletion was covalent, more extensive delipidation with organic solvents was performed (**Fig. 12**). Lanes 1 and 2 compare our standard procedure for delipidating protein precipitates (two washes each with ether–ethanol and

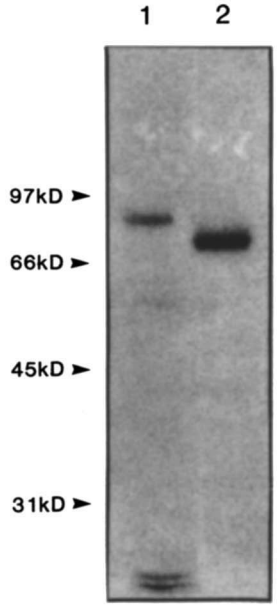

Fig. 5. Digestion of 85-kDa protein with endoglycosidase F. Purified, [^{3}H]palmitate-labeled 85-kDa protein was incubated in the absence (lane 1) and presence (lane 2) of endoglycosidase F.

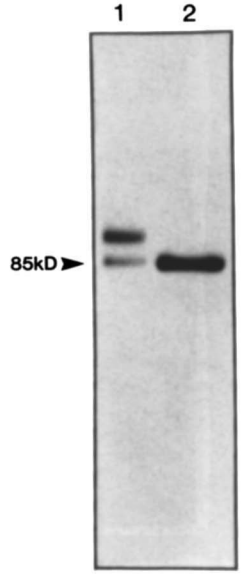

Fig. 6. Susceptibility of 85-kDa protein to cell surface labeling with ^{125}I. Exofacial proteins of intact adipocytes were labeled with ^{125}I using Iodo-Beads. The 85-kDa protein was purified sequentially by wheat germ agglutinin–agarose column chromatography (lane 1) and DEAE-Sepharose ion exchange chromatography (lane 2).

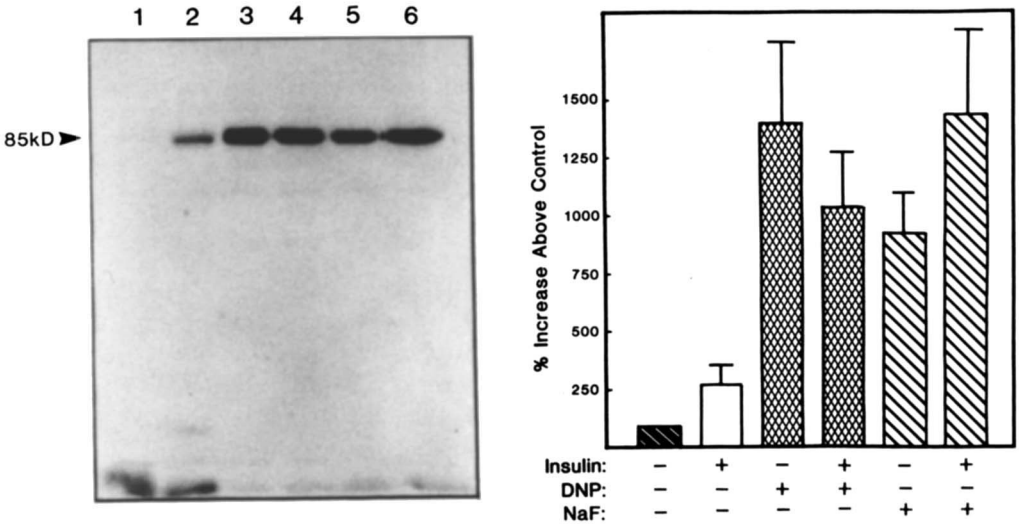

Fig. 7. Effects of insulin and energy depletors on [^{3}H]palmitate labeling of adipocyte proteins. Left panel: adipocytes were incubated for 20 min at 37°C with no additions (lane 1) or with 30 ng/ml insulin (lanes 2, 4, and 6), 1 mM 2,4-dinitrophenol (lanes 3 and 4), or 6 mM sodium fluoride (lanes 5 and 6). [^{3}H]palmitate was added and after a further 60 min incubation the cells were washed and solubilized. The cellular proteins were precipitated, delipidated with organic solvents, and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Right panel: the labeled bands were excised from the gel and quantitated by liquid scintillation counting. Results represent the mean ± SEM for four experiments and are normalized to the counts present in the control (lane 1) incubations.

chloroform–methanol) with more extensive washing. No further label was removed by further treatment with chloroform–methanol.

Fig. 13 and **Fig. 14** show the effects of energy depletion on the time courses of labeling of the 85-kDa protein and extracellular fatty acid pools. In control cells, [^{3}H]palmitate was incorporated into the 85-kDa protein at a low level. Incorporation of label into energy-depleted cells was rapid ($t_{1/2} \sim 15$ min) and maximal by 30 min. The radioactivity of the extracellular fatty acid pool was 7-fold

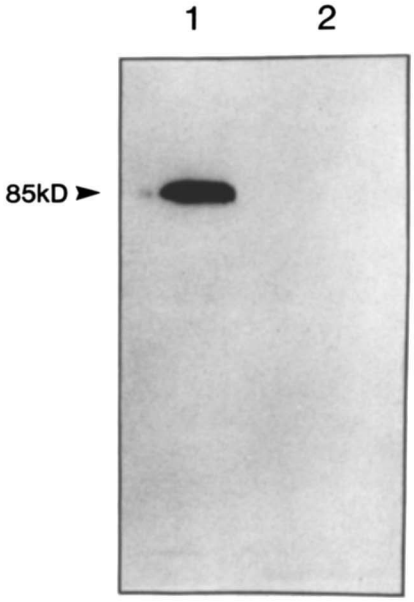

Fig. 8. Sensitivity of [^{3}H]palmitate labeling induced by DNP to removal by neutral hydroxylamine. Adipocytes labeled in the presence of 1 mM DNP were solubilized after which proteins were precipitated and washed with organic solvents. Lane 1: no further procedures. Lane 2: resolubilized proteins were treated with 1 M neutral hydroxylamine prior to SDS-polyacrylamide gel electrophoresis.

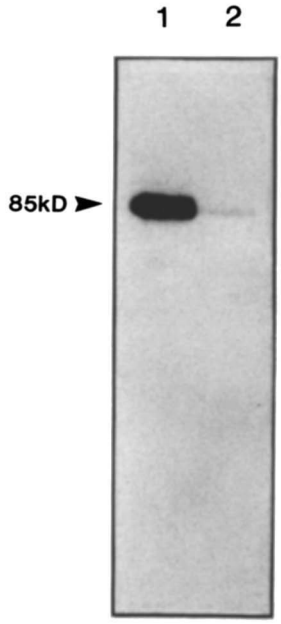

Fig. 9. Effect of temperature on [^{3}H]palmitate labeling induced by DNP. Adipocytes were incubated for 20 min in the presence of 1 mM DNP at 37°C. The cells were divided into two equal batches and further incubated for 1 h with [^{3}H]palmitate at 37°C (lane 1) or 4°C (lane 2).

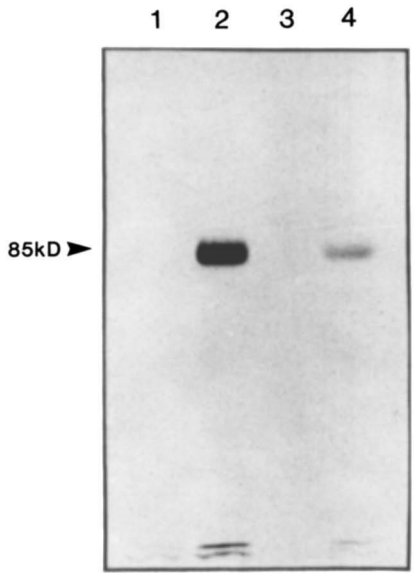

Fig. 10. Effects of N-ethylmaleimide and trypsin on the ability of DNP to induce labeling of the 85-kDa protein. Adipocytes were incubated in the presence (lanes 2–4) or absence (lane 1) of 1 mM DNP for 20 min. At that time, 2 mM N-ethylmaleimide (lane 3) or 150 μg/ml trypsin (lane 4) was added. The cells exposed to trypsin were incubated for 2 min after which 500 μg/ml egg white trypsin inhibitor was added and the cells were washed twice and resuspended in fresh DNP-containing medium. [^{3}H]palmitate was then added to all samples and after a further 45-min incubation at 37°C the cells were washed and solubilized. Adipocyte proteins were precipitated, delipidated, and analyzed by SDS-polyacrylamide gel electrophoresis/autoradiography.

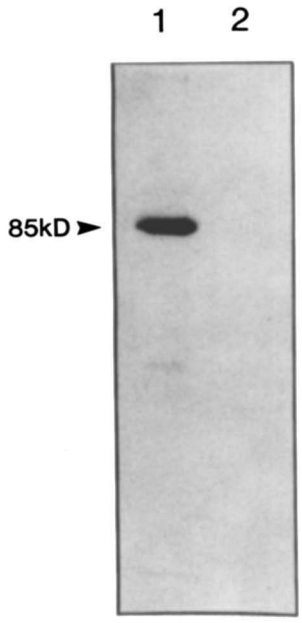

Fig. 11. Effect of cerulenin on the ability of DNP to induce labeling of the 85-kDa protein. Adipocytes were preincubated in the presence (lane 2) or absence (lane 1) of 0.3 mM cerulenin for 90 min prior to the additions of 1 mM DNP and [^{3}H]palmitate.

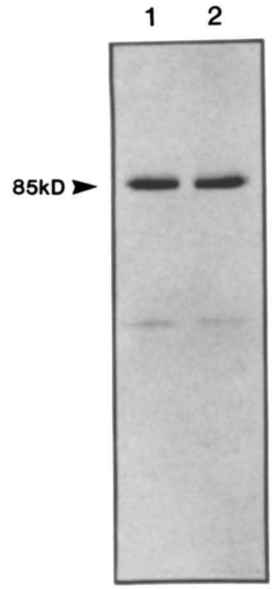

Fig. 12. Effect of extensive delipidation on the linkage between [^{3}H]palmitate and the 85-kDa protein induced by 2,4-dinitrophenol. Adipocytes labeled in the presence of 1 mM 2,4-dinitrophenol were homogenized and ultracentrifuged to produce crude membranes. The membranes were solubilized and membrane proteins were then precipitated with six volumes of ether–ethanol 1:1. Precipitated proteins were washed two times with ether–ethanol 1:1 and twice with chloroform–methanol 2:1 (lane 1) or, alternatively, washed twice with ether–ethanol 1:1, ten times with chloroform–methanol 2:1, and then soaked overnight in chloroform–methanol 2:1 (lane 2). The membrane proteins were then resolubilized and analyzed by SDS-polyacrylamide gel electrophoresis/autoradiography. Approximately 40 μg of membrane protein was electrophoresed in each lane.

higher in treated cells, reflecting inhibition of fatty acid uptake and utilization by energy depletion. Extracellular fatty acid concentration in control cells was approximately 50% higher than in 2,4-dinitrophenol-treated cells.

DISCUSSION

The purpose of this study was twofold. First, to biochemically characterize and identify the major endogenous substrate for long-chain fatty acid acylation in adipocytes; and second, to better define the interactions of the 85-kDa protein with palmitate in order to gain insight into the potential relevance of its acylation to adipocyte physiology.

The 85-kDa protein was successfully purified from adipocyte membranes by lectin affinity chromatography followed by ion exchange chromatography. Its N-terminal sequence was nearly identical to that of CD36 (platelet membrane glycoprotein IV). Although we have yet to determine the full sequence of this protein or to demonstrate immunological identity with CD36, characterization of the acylated 85-kDa protein revealed that it possessed several properties in common with CD36. Specifically,

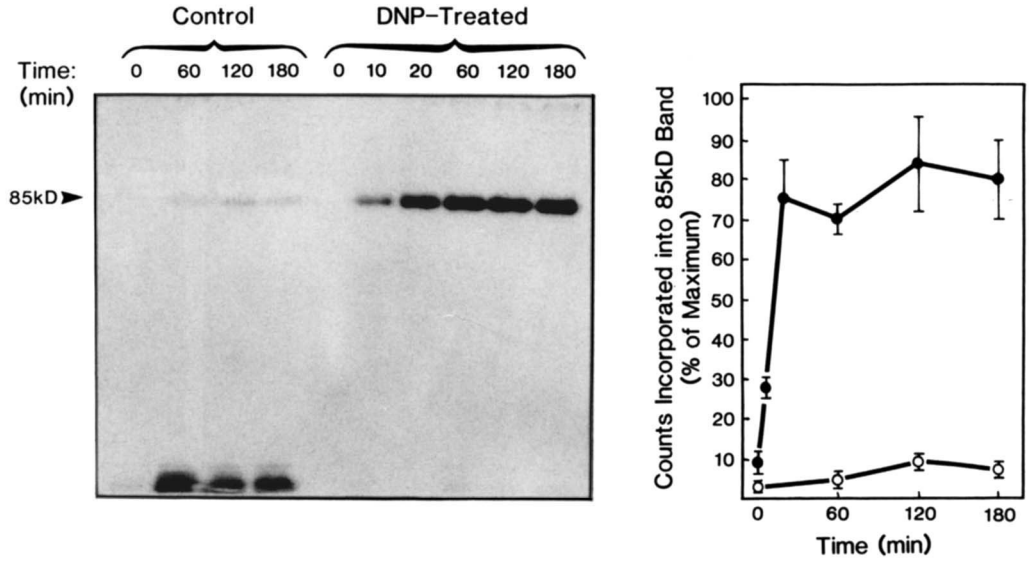

Fig. 13. Time course of protein labeling induced by DNP. Adipocytes were preincubated with 1 mM DNP for 20 min after which [^{3}H]palmitate was added. At the times indicated, cells were solubilized and adipocyte proteins were precipitated and washed with organic solvents. Left panel: SDS-polyacrylamide gel electrophoresis/autoradiography of sample experiment. Right panel: quantitation of labeled 85-kDa band by liquid scintillation counting. Controls, open circles; DNP-treated, closed circles. Results represent mean ± SEM of four experiments.

both CD36 and the acylated adipocyte protein are major membrane glycoproteins, possess extracellular domains, are N-glycosylated, and consist of a single subunit (29). CD36 derives its name based upon its presence as an antigen on the cell surface of differentiated mononuclear cells (30). Similar or identical proteins are found in platelets (glycoprotein IV), endothelial cells, breast epithelial cells, milkfat globule membranes (PAS IV protein), and other cell types (29, 31–33). In addition, lysosomal integral membrane protein II (LIMP II) from rat liver was recently sequenced and shares significant homology with CD36 (34). While CD36 has received particular attention because of its role in the pathogenesis of malaria (35, 36), it also serves as possible receptors for thrombospondin (37) and collagen (38) and has an emerging role in signal transduction in monocytes and platelets. For example, antibodies interacting with an extracellular epitope of CD36 stimulated signal transduction pathways in monocytes (39, 40). In platelets, antibodies recognizing CD36 (platelet membrane glycoprotein IV) co-precipitated *src*-related

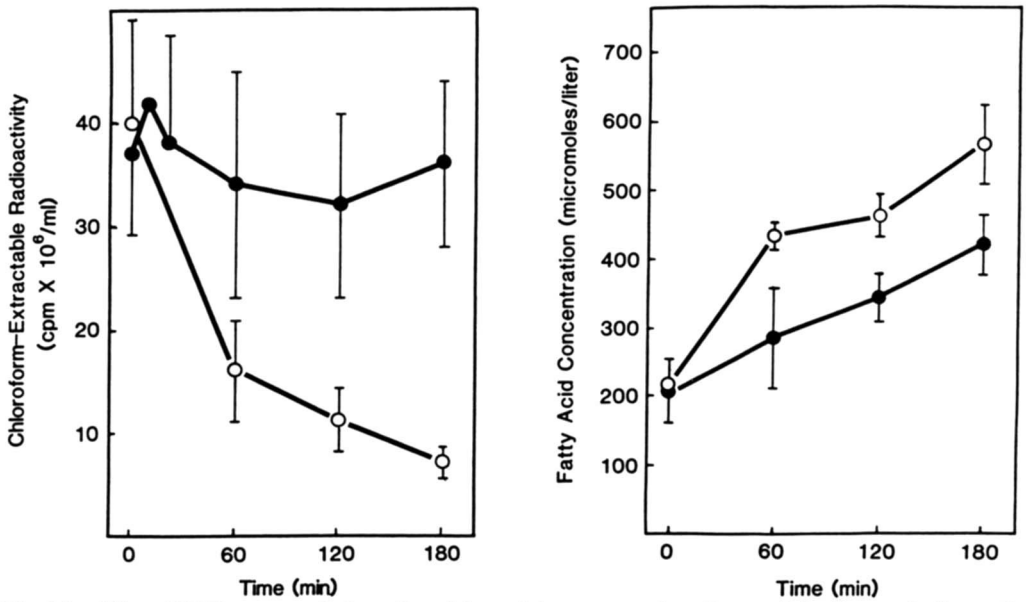

Fig. 14. Effect of DNP on extracellular radioactivity and the concentration of extracellular fatty acids. Extracellular media from the experiments shown in Fig. 13 were assayed for chloroform-extractable radioactivity (panel A) and fatty acid concentration (panel B). Controls, open circles; DNP-treated, closed circles. Results represent mean ± SEM of four experiments.

tyrosine kinases, implying their physical association in the plasma membrane (41).

Membrane and protein trafficking depends upon hydrophobic interactions in order to promote the membrane–membrane and membrane–protein fusion events necessary in vesicle formation and movement. Therefore, our working hypothesis is that a membrane protein, such as the adipocyte 85-kDa protein, present in large quantity and capable of undergoing rapid, dynamic palmitoylation, may prove to be involved in membrane trafficking. Consistent with this hypothesis, we found that cerulenin inhibited both CD36 acylation and membrane trafficking events in adipocytes (42). This notion is further supported by the presence of large amounts of CD36 and related proteins in vesicular structures such as milkfat globules, platelets, and hepatic lysosomes. However, it is possible the 85-kDa protein may serve some other function. Membrane proteins of 85-kDa and 43 kDa have been proposed as candidates for the fatty acid transporter (43, 44). Of particular relevance to the present study, was specific labeling of the 85-kDa candidate transporter by chemical crosslinking fatty acid analogues (45). Therefore, an alternative interpretation of these data is that interaction of fatty acids with the 85-kDa protein represents a covalent, intermediate complex involved in fatty acid uptake. The results are not consistent with the palmitoylated 85-kDa protein being a member of the fatty acid-binding protein family, which are smaller (~15 kDa), cytosolic proteins (46).

An unexpected finding was the marked enhancement of labeling in energy-depleted cells. This was unexpected because of the presumed requirement of protein acylation for metabolic energy. It is generally accepted that intracellular fatty acyl coenzyme A, generated in an ATP-dependent reaction, is the immediate fatty acid donor in protein acylation reactions (47). This raised the possibility that the association between [^{3}H]palmitate and adipocyte CD36 represented unusually strong noncovalent, hydrophobic interactions rather than enzymatic, covalent acylation. We examined this possibility and found that [^{3}H]palmitate labeling of CD36 in energy-depleted cells fulfilled all criteria required of covalent acylation and had properties consistent with an active enzymatic process. The association was stable to extraction by organic solvents and detergents. Furthermore, the label was sensitive to removal by neutral hydroxylamine and labeling was blocked by low temperature and cerulenin.

A more likely explanation for the effects of energy depletion is that the protein–lipid interaction represents true covalent fatty acylation but the acylation has the unusual property of occurring on an extracellular epitope of the 85-kDa protein. This is supported by our demonstration of an exofacial epitope for the 85-kDa protein (Fig. 6) and by our finding that significant labeling of this protein occurred under conditions in which cellular fatty acid utilization was negligible (Fig. 13, 14). Furthermore, the interaction between [^{3}H]palmitate and the 85-kDa protein was sensitive to the inclusion of N-ethymaleimide or trypsin in the extracellular media (Fig. 10). In this scenario, energy depletion enhanced labeling by blocking intracellular [^{3}H]palmitate uptake and utilization, thereby maintaining the availability of extracellular [^{3}H]palmitate to acylate an exofacial epitope. This scenario would imply the presence of deacylated CD36 on the plasma membrane and of a trypsin-sensitive, plasma membrane, protein fatty acyltransferase activity. The existence of plasma membrane acyltransferases has been suggested in previous studies (48).

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