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Journal of Nutritional Biochemistry 13 (2002) 66–74

THE JOURNAL OF Nutritional Biochemistry

Exogenous myristic acid acylates proteins in cultured rat hepatocytes

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Received 28 February 2001; received in revised form 20 July 2001; accepted 7 September 2001

Abstract

Fatty acid acylation is a functionally important modification of proteins. In the liver, however, acylated proteins remain largely unknown. This work was aimed at investigating fatty acid acylation of proteins in cultured rat hepatocytes. Incubation of these cells with [9,10-^{3}H] myristic acid followed by two-dimensional electrophoresis separation of the delipidated cellular proteins and autoradiography evidenced the reproducible and selective incorporation of radioactivity from the precursor into 18 well-resolved proteins in the 10–120 kDa range and the 4–7 pH range. Radiolabeling of these proteins resulted from covalent linkage to the precursor [9,10-^{3}H] myristic acid or to its elongation product, palmitic acid. The majority of the covalent linkages between the proteins and the fatty acids were broken by base hydrolysis, which indicated that the linkage was of thioester or ester-type. Only one of the studied proteins was attached to myristic acid via an amide linkage which resisted the basic treatment but was broken by acid hydrolysis. After incubation with [9,10-^{3}H] palmitic acid, only two proteins previously detected with myristic acid were radiolabeled. Finally, the identified acylated proteins may be grouped into two classes: proteins involved in signal transduction (the α subunit of a heterotrimeric G protein and several small G proteins) and cytoskeletal proteins (cytokeratins, actin).

Keywords: Myristic acid; Palmitic acid; Fatty acid metabolism; Fatty acid acylation of proteins; N-myristoylation; Cultured rat hepatocytes

1. Introduction

Fatty acid acylation of proteins is ubiquitous in living matter [1] and seems to be a functionally important modification of proteins [2]. The functional significance of the acyl moiety may be to mediate protein subcellular localization, protein-protein interaction or protein-membrane interactions required for the expression of the biological activities of many acylated proteins [3]. Fatty acid acylation of eukaryotic proteins has been divided into two classes [4]. Myristoylation (N-acylation) refers to the co-translational covalent attachment of myristic acid, via an amide linkage, to the NH_2-terminal glycine residue of several eukaryotic cellular proteins [5]. Myristoyl-CoA: protein N-myristoyltransferase (NMT, EC 2.3.1.97), the enzyme responsible for this stable acylation, has been characterized in several mammalian tissues and cell lines [6]. Palmitoylation refers to the post-translational addition of long-chain fatty acids (mainly palmitic acid C16:0), via a thioester bond (S-acylation) to cysteine residues of some integral and peripheral membrane proteins [7]. Ester linkage (O-acylation) between fatty acids and serine residues has also been reported [8]. Surprisingly, fatty acid acylation of hepatic proteins remains largely unknown [9], and the importance of exogenous saturated fatty acid supplementation for this function has not been studied. In a previous work [10], we compared the metabolism of myristic acid and palmitic acid in cultured rat hepatocytes in terms of lipid metabolism (incorporation into lipids, β-oxidation, conversion to other fatty acids). In the present work, we further studied myristic and palmitic acid metabolism and investigated fatty acid acylation of proteins in cultured rat hepatocytes. Cells were incubated with radiolabelled myristic acid or palmitic acid. We studied the incorporation of radioactivity from both fatty acids into cellular proteins using two-dimensional (2-D) electrophoresis separation followed by fluorography. In order to detect hepatic acylated proteins, the nature of covalent modification (N- and/or S-linkage) by fatty acids and the identity of these fatty acids were further investigated regarding the well-resolved radio-

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0955-2863/02/$ – see front matter
PII: S0955-2863(01)00196-6

labeled proteins. In addition, tentative identification of many of these proteins was also carried out.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin (BSA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Williams' medium E (W 4125), insulin (bovine), dexamethasone, collagenase, cold fatty acids, polyvinylidene difluoride (PVDF) and nitrocellulose (0.2 μm pore size) membranes were purchased from Sigma (St. Louis, MO). Penicillin-streptomycin antibiotic mixture was provided by Gibco BRL (Eragny, France). Fetal bovine serum was obtained from J. Boy (Reims, France). [9,10-^{3}H] Myristic acid and [9,10-^{3}H] palmitic acid were purchased from DuPont NEN (Le Blanc Mesnil, France). Falcon Primaria Petri culture dishes (60 mm diameter) were used (AES, Combourg, France). CHAPS (3-(3-cholamidopropyldimethylammonio)-1-propanesulphonate), sodium dodecyl sulfate (SDS), Immobiline-DryStrip 4–7L and other reagents for electrophoretical application (PlusOne chemicals) were purchased from Pharmacia Biotech (Orsay, France). Other chemicals were obtained from Merck (Darmstadt, Germany), except high purity reagents for high performance liquid chromatography (HPLC) application, which came from Fisher (Elancourt, France).

2.2. Cultured rat hepatocytes and labeling conditions

Sprague-Dawley male rats (250 g body weight), obtained from the R. Janvier breeding center (Le Genest-St. Isle, France), were food-deprived 12 h prior to liver perfusion. Hepatocytes were obtained by collagenase perfusion in situ, as previously described [10]. The culture medium (Williams'E) was supplemented with 26 mM $NaHCO_3$, 12.5 mM HEPES, 15 μM BSA, antibiotic mixture (50000 IU/L penicillin, 50 mg/L streptomycin), 1 μM insulin and 1 μM dexamethasone. For plating only (1.5×10^6 cells/dish), the culture medium was supplemented with 7% (v/v) fetal bovine serum. After plating, the cells were maintained in a humidified incubator at 37°C under 5% CO_2 in air. After 4 h, the plating medium was changed to a serum-free culture medium. At 24 h of culture, the culture medium was replaced by 1 ml serum-free fresh medium containing [9,10-^{3}H] myristic acid or [9,10-^{3}H] palmitic acid (4 nmol, 7.4 MBq) per dish. Incubation was carried out at 37°C in a 5% CO_2 atmosphere.

2.3. Delipidation and protein electrophoresis

At the end of the incubation, the medium was removed. The cells were washed twice with ice-cold phosphate-buffered saline solution (150 mM NaCl; 5 mM Na Phosphate; pH 7.4) containing 1 mM PMSF (phenylmethylsulfonylfluoride) and 10 μg/ml aprotinin, and harvested with a rubber policeman in the same solution. Cell suspensions were centrifuged (800×g, 4 min, 4°C). The supernatant was discarded and the cell pellet kept for lipid extraction. Lipids which were non-covalently bound to proteins were extracted three times with 4 ml hexane/isopropanol (3:2 v/v) [10]. After each lipid extraction, proteins were separated from the solvent by centrifugation (4000×g, 10 min, 4°C). The final delipidated protein pellet was dried under a stream of N_2 and saved for electrophoretic analysis. The delipidation efficiency was measured by counting the remaining radioactivity in the protein pellet. Depending on the incubation time, the remaining radioactivity represented between 0.01% and 0.05% of the initially added radioactivity. Mono-dimensional SDS-PAGE (12% w/v acrylamide separating gel) were performed according to Laemmli [11]. 2-D electrophoresis was carried out as previously described [12]. Samples (300 μg) were resuspended with sonication in urea-buffer (7 M urea, 2 M thiourea, 4% (v/v) CHAPS, 0.3% (v/v) dithiothreitol, and 2% (v/v) IPG buffer 4–7). After 20 h focusing in the first dimension (48 kVh), IEF (isoelectric focusing) gels were loaded for the second dimension. After staining with Coomassie Blue, gels were treated with Enhance (NEN DuPont, Le Blanc Mesnil, France) for fluorography, dried and then exposed to X-OMAT AR Film (Kodak, Rochester, NY) at -80°C for periods varying from several days to weeks. The radiolabeled spots corresponding to the resolved proteins were localized by superimposing fluorographs and the original gels.

2.4. Release and identification of fatty acids covalently bound to proteins

Following 2-D gel, proteins were electroblotted on nitrocellulose membrane in a semidry electroblotter (Ancos, Denmark) using glycine 39 mM, SDS 1.3 mM, methanol 20% (v/v), Tris 48 mM, pH 8.4 as buffer. The resolved Ponceau Red-stained proteins corresponding to the previously detected radiolabeled spots were excised from the membrane and washed three times with 1 ml chloroform/methanol (2:1 v/v) to remove any remaining non-covalently bound radiolabeled lipids. Five pieces of nitrocellulose containing the same protein were pooled for subsequent fatty acid analysis [13]. To release thioester- and ester-linked fatty acids, the protein was first saponified in 1 ml of 2 M KOH in ethanol at 70°C for 4 h under nitrogen. Following addition of 1 ml HCl 3 N and 4 ml H_2O, liberated fatty acids were extracted twice with 4 ml diethylether. The ether extracts were combined and kept for chromatographic analysis, as described below. The subsequent release of the possibly remaining amide-linked fatty acids was realized by adding 4 ml HCl 12 N to each tube followed by hydrolyzation at 100°C for 15 h under nitrogen. Released fatty acids were then extracted twice with 4 ml diethylether.

Fatty acids released from each protein by these two successive treatments were converted to fatty acid naphtha-

cyl esters and separated on HPLC (Alliance Integrated System, Waters, St Quentin en Yvelines, France) as recently described [14]. Elution of naphthacyl derivatives was monitored by UV absorbance at 246 nm (tunable absorbance detector 486, Waters). Peaks corresponding to radiolabeled fatty acids were collected and subjected to liquid scintillation counting (Packard Tri-Carb 1600 TR, Meriden, CT). Preliminary identification of fatty acid naphthacyl esters was based upon retention times obtained for naphthacyl derivatives prepared from fatty acid standards.

2.5. Amino acid composition of proteins and peptide mass spectrometry

Amino acid composition (AAC) of proteins was determined under standard conditions. Following 2-D gels, proteins were electroblotted on PVDF membranes, stained with Coomassie Blue and destained in warm 50% ethanol. The proteins of interest were excised from the membrane and washed five times in an aqueous solution to remove the traces of glycine carried over from SDS-PAGE. Pieces of PVDF blot were sealed in vacuo in glass tubes and hydrolyzed in 6 N HCl and 0.1% phenol for 24 h at 120°C. Amino acids were derivatized with phenylisothiocyanate (quantified as phenylthiocarbonyl-amino acids) and were established on an Applied Biosystem 420A derivatizer [12].

In situ enzymatic digest of proteins [15] blotted on nitrocellulose membranes was carried out with the use of endoproteinase Arg-C (Boehringer, Mannheim) according to the procedure supplied by the producer (15 pieces of nitrocellulose containing the same protein were pooled). Generated peptides were fractionated on a Vydac C18 reverse-phase column (Western Analytical products, Murrieta, CA) and collected. Peptides were then analyzed by MALDI-TOF (matrix assisted laser desorption/ionization-time of flight) mass spectrometry using a reflectron Voyager DE mass spectrometer (Perseptive Biosystem, Boston, MA). The mass range was calibrated using bovine insulin (average molecular mass 5734.6 Da) and a matrix peak (379.1 Da) as internal standards. Samples were dissolved in 0.1% trifluoroacetic acid (TFA) at 10 pmol/μl. One μl was applied to a sample slide and allowed to air-dry, before applying 1 μl of α-cyano-4 hydroxycinnamic acid (10 mg/ml) in ethanol/acetonitrile/0.1% TFA 1:1:1 (v/v/v). The matrix was allowed to air-dry before collecting spectra. Mass spectra were generated from the sum of 50 laser spots.

2.6. Database searching and protein identification

The algorithms of the amino acid composition versus protein identity (AAC-PI) method and the structure of AAC database have been described in detail [16]. The PIR and PATCHX protein databases containing 231071 sequences were used for creation of the parameters and coding files. Comparisons between experimentally established AACs (16 amino acids), mass of proteins (± 5 kDa) established using protein standards and pIs of proteins (± 2) established experimentally, versus those which are stored in the parameter file were performed by the AMICOM library of algorithms. Each AMICOM analysis supplies a list of candidate proteins whose AACs match the AAC of the investigated protein. The candidate proteins are arranged according to increasing values of root-mean-square difference (Rmsd) which is calculated as previously described [16]. Only the proteins whose AACs converge to coherent groups (converge to a specific family of proteins) are considered to be unique. Usually a Rmsd between 0.5 and 1.4 indicates that the AAC is unique.

The PIR protein database was also used for the search of protein identity corresponding to peptide masses obtained with MALDI-TOF-MS. Input parameters consisted of a list of observed peptide masses (with a mass tolerance), the protease used for proteolytic cleavage, the protein molecular mass and an approximated pI. Each analysis supplies a list of candidate proteins whose peptide masses match the experimentally obtained peptide masses of the investigated protein. Only the proteins whose peptide masses converge to coherent groups are considered to be unique.

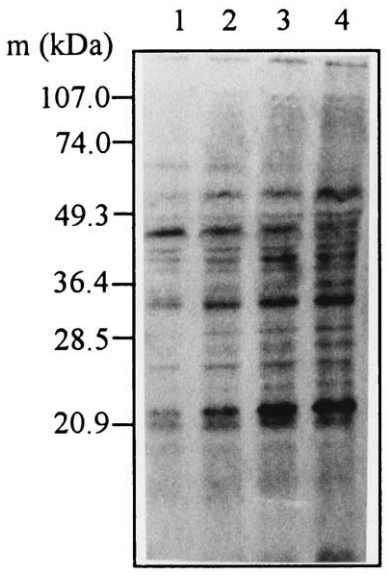

Fig. 1. ^{3}H incorporation from [9,10-^{3}H] myristic acid into proteins of cultured rat hepatocytes. Cells were incubated for 3 h (lane 1), 6 h (lane 2), 12 h (lane 3) and 24 h (lane 4) with [9,10-^{3}H] myristic acid (4 nmol and 7.4 MBq per dish). After each of these incubation times, the cellular delipidated proteins were subjected to SDS-PAGE (12% w/v acrylamide separating gel) followed by fluorography. Each lane was loaded with 500 μg of protein. Gels were exposed to X-OMAT AR film at -80°C for 5 days.

3. Results

3.1. ^{3}H incorporation from [9,10-^{3}H] myristic acid into cellular proteins

In order to detect the acylated proteins, rat hepatocytes at 24 h of culture were incubated with [9,10-^{3}H] myristic acid

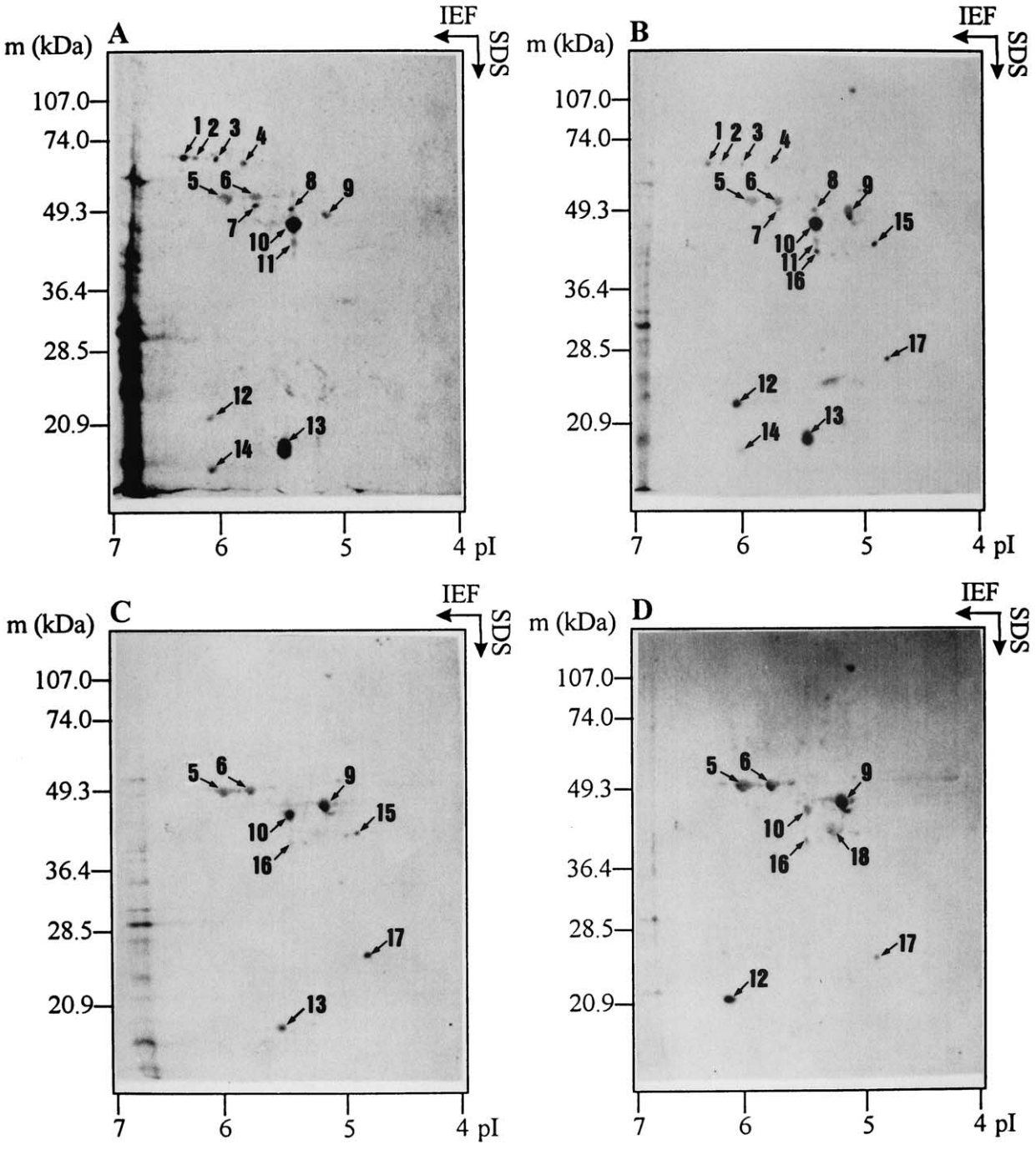

Fig. 2. ^{3}H incorporation from [9,10-^{3}H] myristic acid into proteins of cultured rat hepatocytes. Cells were incubated for 3 h (A), 6 h (B), 12 h (C) and 24 h (D) with [9,10-^{3}H] myristic acid (4 nmol and 7.4 MBq per dish). After each of these incubation times, the cellular delipidated proteins were subjected to two-dimensional electrophoresis (IEF 4–7 followed by 12% w/v acrylamide separating gel) followed by fluorography. Each gel was loaded with 300 μg of protein. Gels were exposed to X-OMAT AR film at -80°C for 20 days.

for 3, 6, 12 and 24 h. After each of these incubation periods, the delipidated cellular proteins were subjected to mono-dimensional (Figure 1) and 2-D electrophoresis (Figure 2) followed by fluorography. The radioactivity appeared to be selectively and reproducibly (3 separate experiments were performed) incorporated into 15 to 25 proteins, depending on the incubation time (Figure 1). On 2-D gels (Figure 2), two major radiolabeled proteins (spots 10 and 13) appeared at 3 h, with approximate mass and pI of 45 kDa, 5.5 and 20 kDa, 5.5, respectively. The radioactivity of these 2 proteins seemed to decrease with time. On the contrary, the label of protein 9 increased with time and protein 18 appeared to be labeled only after 24 h of incubation. Similar experiments were carried out with [9,10-^{3}H] palmitic acid (Figure 3).

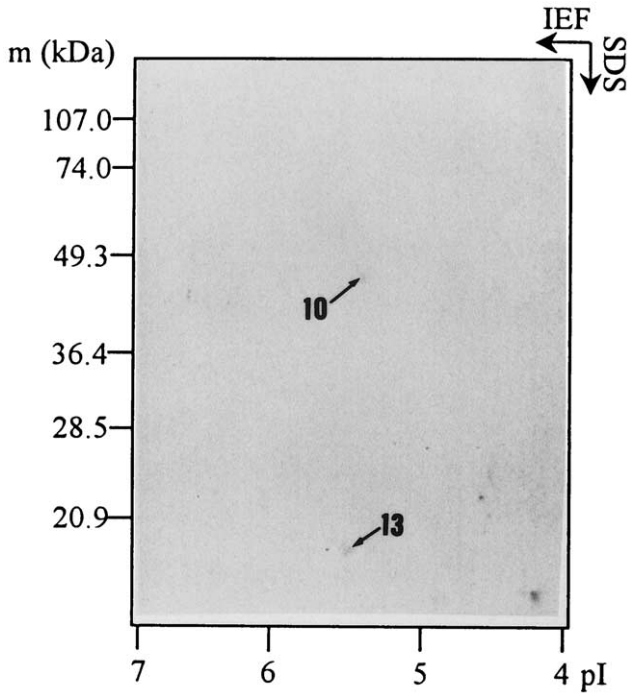

Fig. 3. ^{3}H incorporation from [9,10-^{3}H] palmitic acid into proteins of cultured rat hepatocytes. Cells were incubated for 3 h with [9,10-^{3}H] palmitic acid (4 nmol and 7.4 MBq per dish). Otherwise, same legend as in Figure 2.

They revealed that, whatever the incubation time, only a small number of proteins were selectively and reproducibly (3 experiments) labeled after incubation with palmitic acid. Spots 10 and 13 which were previously detected with myristic acid were also weakly radiolabeled after incubation with palmitic acid.

3.2. Identification of fatty acids released from radiolabeled proteins

Since the presence of radioactivity (Figure 2) resisted successive extensive delipidation, urea-denaturation, 2-D electrophoresis and staining procedure (including methanol washing), it was likely that the radioactivity was attached to some proteins via covalent linkages. In order to determine the kind of linkage between the radiolabel and the proteins, most of the previously well-resolved detected proteins (after 2-D electrophoresis and western blotting followed by spot excision) were submitted to alkaline hydrolysis followed by acid hydrolysis to release the possibly S- (or O-) and N-linked fatty acids respectively. Identification of the released radiolabeled fatty acids was achieved by HPLC analysis. HPLC profiles for radiolabeled fatty acids released by alkaline and acid hydrolysis of the proteins are shown in Figure 4. The major part of the radioactivity was recovered and identified as labeled fatty acids. The small amounts of radioactivity observed sometimes in the early phase of the profiles (Figure 4) could be attributed to the degradation of the fatty acids during chemical treatment.

Only one protein, protein 10 (Figure 4A), exhibited an amide linkage with myristic acid, which was released by acid hydrolysis and not by the initial alkaline treatment. Indeed, base hydrolysis of protein 10 showed that labeled myristic acid but also palmitic acid, presumably formed endogenously through the elongation of the labeled precursor, were covalently bound via alkali-labile thioester or ester linkage. Subsequent acid hydrolysis showed that only C14:0 was bound via an acid-labile linkage. This result showed that this protein was both N-acylated with myristic acid and S-acylated with myristic and palmitic acids. All the other studied proteins exhibited a thioester or ester linkage to the precursor myristic acid and/or to its elongation product, palmitic acid. For instance, after 3 h incubation with myristic acid, protein 13 (Figure 4B) was acylated with almost solely alkali-labile (S- or O-acylated) myristic acid. Protein 14 (Figure 4C) was primarily acylated with exogenous alkali-labile myristic acid and also with a small amount of endogenous palmitic acid. The 4th studied protein, protein 1 (Figure 4D), exhibited linkages to both fatty acids (the exogenous myristic acid and the endogenous palmitic acid) by thioester or ester bonds, palmitic acid being the most abundant. Protein 5 (Figure 4E) showed primarily alkali-labile endogenous C16:0. Figure 4F presents the profile of fatty acids released on protein 18, which appeared only after 24 h incubation with myristic acid. Base hydrolysis of protein 18 released small amount of radiolabeled myristic acid.

3.3. Tentative identification of acylated proteins

Tentative identities of the proteins (after western blotting followed by spot excision) were first established using their AACs. Listed in Table 1 are proteins identified by spot number that correspond to 2-D gels shown in Figure 2. The AACs of many proteins were sufficiently unique (small Rmsd) to allow establishment of protein identity with a relative high degree of confidence. In certain cases, molecular mass of peptides were obtained after in situ digestion with Arginine-C protease with MALDI-TOF mass spectrometry and confirmed some of our identifications (Table 2).

AAC of the protein 10 allowed us to assign it to the α-subunit of a heterotrimeric G_0 protein. Amino acid analysis of the proteins 12, 13, 14 and 17 indicated that these proteins were related to the small G protein family. Protein 18 was identified as β-actin. This protein was also unequivocally identified by MALDI-TOF-MS analysis (Table 2) and by immunoblotting (not shown). Analyses of the AACs of the proteins 5, 6 and 9 (Table 1), and MALDI- TOF-MS results (Table 2) revealed that they are type II and type I cytokeratin, respectively.

4. Discussion

The purpose of this work was to investigate fatty acid acylation of proteins in cultured rat hepatocytes. 2-D elec-

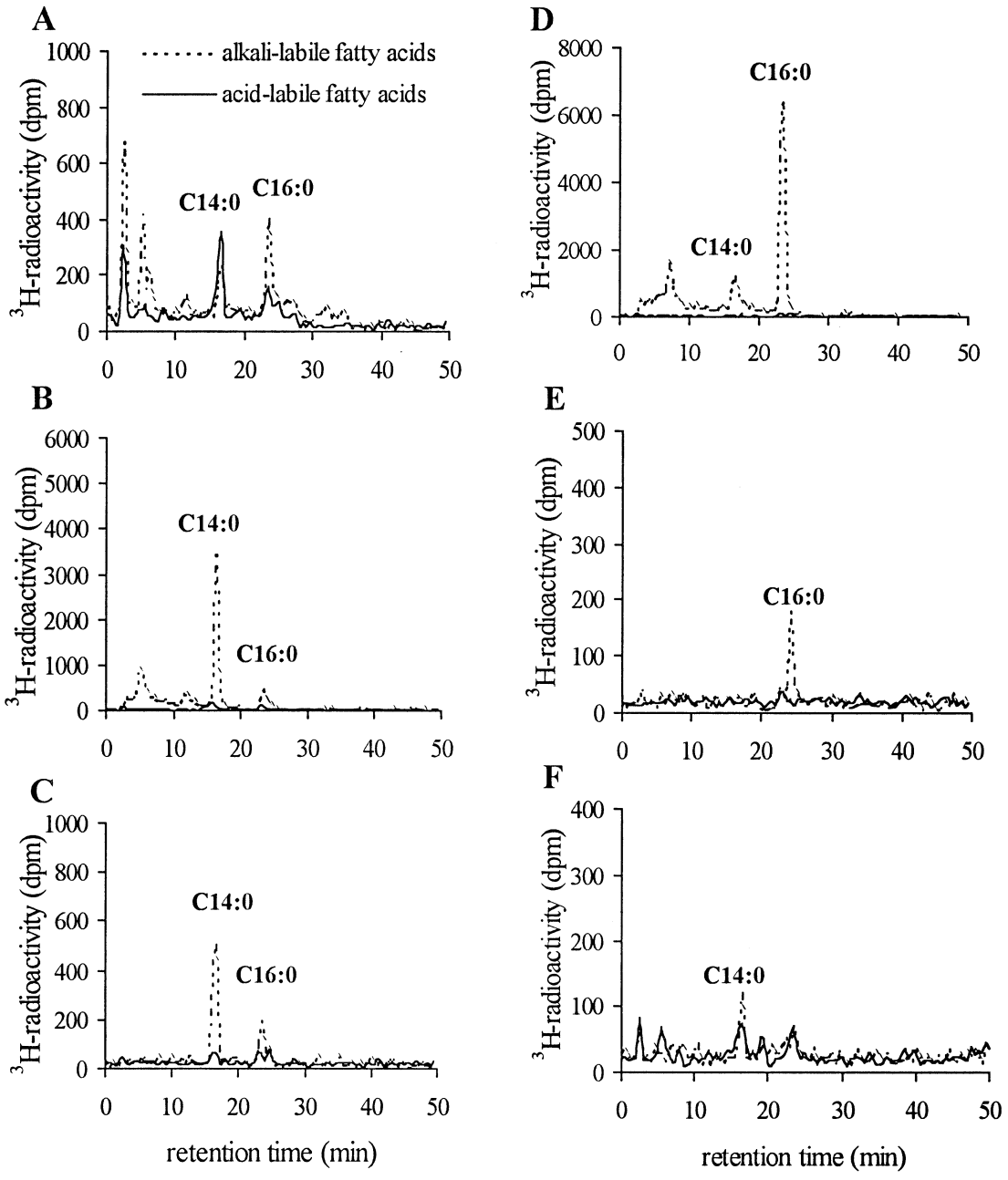

Fig. 4. HPLC elution profiles of radiolabeled fatty acids released by successive alkaline and acid treatment of several radiolabeled proteins (A: protein 10; B: protein 13; C: protein 14; D: protein 1; E: protein 5; F: protein 18). After incubation for 3 h or 24 h with [9,10-^{3}H] myristic acid (4 nmol and 7.4 MBq per dish), 2-D electrophoresis and western blotting followed by spot excision, each protein was submitted to alkaline hydrolysis followed by acid hydrolysis to release the S- (or O-) and N-linked fatty acids respectively. Identification of the fatty acids was achieved by HPLC analysis of each extract. This figure is a representative example of three independent hepatocyte preparations.

trophoresis was used as a preparative method to separate the proteins, because recent improvements in 2-D electrophoresis, amino acid compositions and mass spectrometry, together with algorithms for searching sequence databases, present the opportunity to characterize cellular proteins in small amounts [13,17] and to identify the fatty acids bound to each previously detected protein.

The results first show a reproducible and selective incorporation of radioactivity from myristic acid into several proteins (Figure 2), although only a minor part of the initially added radioactivity is directed to the acylation of proteins. The presence of fatty acylated proteins in cultured rat hepatocytes provides evidence that the enzymes, which catalyze the fatty acid acylation of proteins, are expressed and can be studied in this model. The data reported in the literature show that hepatocytes in primary culture have not been used for studying this fatty acid function. Myristoyl-CoA: protein N-myristoyltransferase (NMT) activity has

Table 1
Tentative identities of acylated proteins from cultured rat hepatocytes using the AAC-PI method

Spot n°	Rmsd	Identified protein	Library code	Protein characteristics	
				M_m (kDa)	pI
5	1.121	Keratin, type II cytoskeletal 4	K2C4_HUMAN	57.3	6.2
6	1.105	Keratin type II, microfibrillar (sheep)	KRSHL2	55.2	6.0
9	0.946	Keratin, type I, cytoskeletal (mouse)	I59463	47.5	5.1
10	1.220	GTP-binding regulatory protein G_0, α-2 chain (rat)	RGRT02	40.1	5.6
12	1.149	Ras-homolog GTPase rab4b (rat)	S58279	23.6	5.7
13	1.170	GTP-binding protein ram (rat)	S12959	25.1	5.0
14	1.383	Small GTP-binding protein rab25 (rabbit)	A48500	23.4	6.0
17	1.224	Rab protein 30 (human)	JC4962	23.0	4.9
18	0.882	Actin β (rat)	ATRTC	41.8	5.2

Following 2-D gels, proteins were blotted on PVDF membranes. The proteins of interest (spot numbers correspond to gels shown in Fig. 2) were excised from the membrane and analyzed using the AAC-PI method as described in Materials and methods. It was possible to establish the identities of the listed proteins since their amino-acid compositions are quasi-unique, as shown by the calculated Root-mean-square differences (Rmsd).

been detected in rat liver ex vivo [18]. The hepatic activity was found to be significantly lower than in other tissues, particularly less than in the brain [19]. Apart from the retina [20], N-myristoylation is generally considered to be highly specific for myristoyl-CoA [21], and S-acylated proteins have been described to be primarily attached to palmitic acid [22]. However, in bovine liver, it has recently been shown [9] that proteins were S-linked to C16:0 (30%), C18:0 (46%) and C18:1 n-9 (9%), and that they were N-linked to C18:0 (45%), C14:0 (27%) and C16:0 (26%).

In our results, the majority of the covalent linkages between the proteins and the fatty acids were broken by base hydrolysis (Figure 4), which indicates that the linkage is of thioester or ester-type. Despite intense recent investigation [23], the enzymes involved in S-acylation and/or O-acylation have not yet been clearly identified and a non-enzymatic process has also been proposed [24]. The protein 10 only (Figure 2) is attached to myristic acid via a linkage which resists the basic treatment but is broken by acid hydrolysis (Figure 4A), which indicates that the linkage is an amide-type linkage, showing that NMT is still active in cultured rat hepatocytes.

Since the enzymes involved in protein acylation are not well characterized, it is interesting to determine the cellular fate of exogenous myristic acid (and elongation products) as substrate for the acylation of proteins. Our results showed that the radiolabeled patterns obtained with myristic acid (Figure 2) and with palmitic acid (Figure 3) are different. Eighteen proteins were radiolabeled after incubation with myristic acid whereas only 2 proteins (which are also visualized after incubation with myristic acid) appeared after incubation with palmitic acid. Differences in the radiolabeled patterns after incubation with myristic and palmitic acid have also been shown in BC_3H1 murine muscle cells

Table 2
Tentative identifies of acylated proteins from cultured rat hepatocytes using MALDI-TOF mass spectrometry

Spot n°	Identified protein	Library code	Protein characteristics		Peptide mass (Da)	ΔDa	Peptide sequence consistent with mass	Residues n°
			M_m (kDa)	pI				
5	Cytokeratin EndoA (mouse)	JS0648	54,6	5,6	1053,3	+0,2	R/QIHEEEIR/	232–239
					1060,4	+0,3	R/KLLEGEESR/	399–407
					913,3	+0,3	R/VGSSSSSFR/	41–49
					879,2	+0,3	R/SFTSGPGAR/	24–32
9	Keratin 19, type I, cytoskeletal (human)	KRHU9	44,1	4,9	1554,7	+0,1	R/QSSATSSFGGLGGGSVR/	8–24
					1041,6	+0,4	R/IVLQIDNAR/	151–159
					909,0	+2,0	MTSYSYR/	1–7
					818,9	−1,8	R/SEVTDLR/	294–300
					1827,6	−0,5	R/FGPGVAFRAPSIHGGSGGR/	25–43
18	Actin β (rat)	ATRTC	41,8	5,2	2507,3	−0,5	R/HQGVMVGMGQKDSYVGDEAQSKR/	40–62
					1132,3	+0,1	R/GYSFTTTAER/	197–206

Following 2-D gels, proteins were blotted on nitrocellulose membranes. The proteins of interest (spot numbers correspond to gels shown in Fig. 2) were excised from the membrane. In situ enzymatic digest of protein was made using Arg-C protease. Generated peptides were analyzed by MALDI-TOF mass spectrometry as described in Materials and methods. ΔDa represents the difference between the experimental mass and the calculated mass.

[25], in several epithelial cell lines [26] and in human keratinocytes [27].

In our model, myristic acid can easily substitute for palmitic acid in S- and/or O-acylation. We also found many proteins acylated with endogenous palmitic acid after incubation with myristic acid (Figure 4), although these proteins were not acylated by exogenous palmitic acid (Figure 3). As was also proposed in the nematode [28], the origin and cellular pathways of fatty acids may influence their utilization for protein acylation. Finally, as already observed by analyzing total liver proteins [9], we did not find any radiolabeled C14:1 n-9 and/or C14:2 n-6 (coming from putative successive desaturations of C14:0), attached to the hepatocyte proteins.

The number of known myristoylated proteins in the liver is limited, to our knowledge, to one protein. In bovine [29] and rat liver [30], NADH-cytochrome b5 reductase has been shown to contain covalently bound myristic acid. NADH-cytochrome b5 reductase requires myristic acid for association with outer mitochondrial membrane but not with endoplasmic reticulum membranes. This protein is, however, not recovered after 2-D electrophoresis separation (Figure 2) since the range of pH is limited to 4–7. After 2-D electrophoresis separation, our results showed that the α subunit of a heterotrimeric G protein is N-terminally myristoylated (amide linkage) in cultured rat hepatocytes, and that several cytoskeletal proteins (actin, cytokeratin) and several small G proteins are S- or O-acylated (thioester or ester linkage) with myristic acid and palmitic acid (Tables 1 and 2).

The described acylation of actin and cytokeratins type II (Tables 1 and 2) could be an S-acylation because they contain several putative palmitoylable cysteine residues. In a recent study [31], it has been shown that chemical S-acylation of commercial actin appeared when the protein was incubated with [^{3}H]-palmitoyl-CoA, suggesting a non-enzymatic S-acylation in vivo. However, actin from *Dictyostelium discoideum* has been reported to be acylated with palmitic acid in vivo [32]. After incubation with ^{3}H-palmitate, keratin polypeptides obtained from mouse mammary epithelial cells were also found to be modified by covalent attachment of lipids [33]. Therefore, these proteins could join the growing list of cytoskeletal proteins such as vinculin, ankyrin [3] and tubulin [34] that are already known to be palmitoylated. The α-subunit of several heterotrimeric G proteins are known to be N-acylated with C14:0 and sometimes also S-acylated with C16:0 [35]. These acylations are required for the function of G proteins which are involved in signal transduction [35]. We demonstrated, in the present work, that G protein α-subunit (protein 10) was both myristoylated and palmitoylated in rat hepatocytes (Table 1). Several small G proteins are also known to be firstly prenylated and then S-acylated [36], the latter seemed also to be the case in our model (proteins 12, 13, 14 and 17). Based on their function, the identified proteins may be grouped into two classes: proteins involved in signal transduction (the α subunit of heterotrimeric G protein and small G proteins) and cytoskeletal proteins (cytokeratins, actin).

Fatty acid acylation of many proteins seems to be required for their function. Therefore, the present study emphasizes the major role of exogenous (dietary) myristic acid for protein activation and consequently for cell function regulation in the liver.

Acknowledgments

The authors are indebted to ARILAIT RECHERCHES (France) for constructive scientific discussion and financial support. Financial support for this research was also provided by the Conseil Régional de Bretagne.

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