Studies on the cellular location, physical properties and endogenously attached lipids of acylated proteins in human squamous-carcinoma cell lines

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The location of cell proteins with covalently attached lipid was examined in two human squamous-carcinoma cell lines. Cells were labelled with either palmitic acid or myristic acid and disrupted by sonication, followed by differential centrifugation of the cell lysates. SDS/polyacrylamide-gel electrophoresis of the resulting cell fractions indicated that most of the palmitate-labelled proteins were found in cell membranes, whereas most of the myristate-labelled proteins were found in the cytosol, although some were located in cell membranes. Experiments with lipid-labelled proteins extracted with the phase-separable detergent Triton X-114 showed that palmitate-labelled proteins behaved as hydrophobic proteins, partitioning into the lower phase of the detergent, whereas most of the myristate-labelled proteins remained in the upper phase. Although one of these cell lines expressed large amounts of epidermal-growth-factor receptor, this could not be labelled by either myristic acid or palmitic acid, whereas transferrin receptor was labelled by palmitic acid. The lipids normally attached to cell proteins in these two human squamous-carcinoma cell lines were characterized by labelling the cells with [³H]acetate. The labelled cell proteins were exhaustively extracted with organic solvents, and subjected to sequential alkaline and acid hydrolyses to release the attached lipids, which were then analysed by h.p.l.c. Most of the lipid released by the alkaline treatment chromatographed as palmitic acid or stearic acid, whereas the subsequent acid treatment released myristic acid as well as some palmitic acid and stearic acid. No other fatty acids apart from these were found attached to cell proteins.

INTRODUCTION

It has been recognized that proteins may undergo post-translational modification by the covalent attachment of lipid, as well as the more common modifications of glycosylation, proteolytic cleavage or phosphorylation. The covalent attachment of lipids to proteins was first described for viral coat proteins (Schmidt *et al.*, 1979; Schmidt & Schlesinger, 1979, 1980), but has also been reported for cell proteins as diverse as transferrin receptor (Omary & Trowbridge, 1981), cardiac muscle cyclic-AMP-dependent protein kinase (Carr *et al.*, 1982), the Ca²⁺-binding subunit of calcineurin B (Aitken *et al.*, 1982), human histocompatibility antigens (Kaufman *et al.*, 1984), rhodopsin (O'Brien & Zatz, 1983), apolipoprotein A-1 (Hoeg *et al.*, 1986), cytochrome *b*₅ reductase (Ozols *et al.*, 1984), ankyrin (Staufenbiel & Lazarides, 1986) and the α - and β -subunits of the nicotinic acetylcholine receptor (Olson *et al.*, 1984).

Interest in this post-translational modification of proteins has been strengthened by the observations that several viral transforming proteins have covalently attached lipid. These include p60^{src} of Rous-sarcoma virus (Sefton *et al.*, 1982; Garber *et al.*, 1983), the large T antigen of simian virus 40 (Henning & Lange-Mutschler, 1983; Klockmann & Deppert, 1983), p21 of Harvey-sarcoma virus (Sefton *et al.*, 1982) and gp120 of Aleson virus (Sefton *et al.*, 1982). The observations that

mutants of the transforming proteins p21 and p60^{src} that lack the attached lipid are transformation-defective and no longer associate with the plasma membrane (Garber *et al.*, 1983; Willumsen *et al.*, 1984; Kamps *et al.*, 1985) serve to underline the importance of the attachment of lipid to these proteins for the full expression of their transforming potential.

These results prompted us to investigate the ability of both normal human keratinocytes and cell lines derived from human squamous carcinomas to attach lipids to protein. Our results showed that these cells could indeed attach myristic acid and palmitic acid to selected populations of proteins, and that different protein populations were labelled by each of the fatty acids (McIlhinney *et al.*, 1985). Furthermore, the attachment of myristic acid was prevented by the protein-synthesis inhibitor cycloheximide, whereas the attachment of palmitic acid was not, suggesting that there were two independent pathways for the attachment of the two lipids to cell proteins. These results have since been confirmed by studies on other cell types (Magee & Courtneidge, 1985; Olson *et al.*, 1985), and in the present work we have continued our investigations by studying the cellular location of the proteins labelled by myristic acid and palmitic acid, and their behaviour in the phase-separable detergent Triton X-114. The possibility that epidermal-growth-factor (EGF) receptor might have attached lipid was also examined.

Abbreviation used: EGF, epidermal growth factor.

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In all of the investigations of the attachment of lipid to protein, including those referred to above, the cells were labelled by the addition of radioactive palmitic acid or myristic acid, and the possibility that other fatty acids might be the preferred substrates for attachment to proteins was not examined. We have therefore labelled two human squamous-carcinoma cell lines with [^3H]acetate and have examined the endogenous lipids attached to cell proteins by h.p.l.c. The results described below show that the main fatty acids attached to proteins in these cells are indeed myristic acid, palmitic acid and stearic acid, with no detectable contributions from other fatty acids.

MATERIALS AND METHODS

Materials

[^3H]Palmitic acid (30 Ci/mmol) and [^3H]myristic acid (13 Ci/mmol) were obtained from New England Nuclear, and sodium [^3H]acetate (3 Ci/mmol) and [^{35}S]methionine (800 Ci/mmol) were supplied by Amersham International. Lipid standards were obtained from Sigma Chemical Co., and all other chemicals unless otherwise specified were of AnalaR quality and were purchased from BDH Chemicals.

Cell lines

The cell lines used in this study, LICR-LON HN5 and LICR-LON HN6, were derived from human squamous carcinomas of the head and neck (Easty *et al.*, 1981). They were maintained in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% (v/v) foetal-calf serum containing penicillin (100 units/ml), streptomycin (100 units/ml) and minocyclin (1 $\mu\text{g}/\text{ml}$). The buffer used for washing the cells after labelling with either radioactive fatty acids or acetate was 50 mM-Tris/HCl buffer, pH 7.2, containing NaCl (0.1 M) and MgCl_2 (1 mM), referred to below as TNM buffer.

Labelling of cells with ^3H -labelled lipids, [^3H]acetate and [^{35}S]methionine

Cells in 25 cm² flasks were labelled with the radioactive lipids at 20 $\mu\text{Ci}/\text{ml}$ in Dulbecco's modified Eagle's medium containing 5% (v/v) dialysed foetal-calf serum. Cells in 75 cm² flasks were labelled with [^3H]acetate (25 $\mu\text{Ci}/\text{ml}$) by incubation in normal culture medium for 36–48 h. The cells were washed, and the cell proteins were extracted with detergent, precipitated with acetone and prepared for SDS/polyacrylamide-gel electrophoresis or for lipid extraction as previously described (McIlhinney *et al.*, 1985).

The precipitated proteins were extracted three times with chloroform/methanol (2:1, v/v), twice with chloroform/methanol (1:1, v/v), once with acetone, twice with ethanol and once with methanol. By this time no detectable radioactivity was released from the protein pellets. The remaining radioactivity bound to the proteins was released by treating them with 0.1 M-KOH in methanol for 30 min at 37 °C with agitation. The reaction mixture was neutralized with 1 M-HCl, and the proteins were pelleted by centrifugation (30000 g-min) and then extracted twice with methanol. These methanol washes were pooled with the hydrolysate supernatant and extracted with light petroleum (b.p. 40–60 °C) to yield the total lipid released from the proteins by alkali hydrolysis. The cell pellet was then further extracted by

treatment with 3 M-HCl in methanol at 90 °C for 6 h, followed by extraction of the released material with light petroleum (b.p. 40–60 °C) to yield the lipids bound to protein and removed by acid hydrolysis. These sequential hydrolysis conditions were more severe than those used previously, which were found to release more than 90% of the protein-bound lipid from cells incubated with radioactive fatty acids (McIlhinney *et al.*, 1985).

To determine the relative concentrations of the total fatty acids in cells labelled with [^3H]acetate, the cells were scraped from the culture dishes and subjected to the exhaustive organic-solvent extraction described above. The extracts were pooled, rotary-evaporated and hydrolysed in 0.1 M-NaOH in 90% (v/v) methanol for 30 min at 37 °C. The hydrolysate was neutralized with 0.1 M-HCl and extracted three times with hexane. The extracts were pooled, concentrated by rotary evaporation, and analysed for their fatty acid content by h.p.l.c. as described below.

Cells were labelled with [^{35}S]methionine (20 $\mu\text{Ci}/\text{ml}$) in methionine-free Dulbecco's modified Eagle's medium containing 10% foetal-calf serum that had been dialysed against phosphate-buffered saline (0.136 M-NaCl/2.6 mM-KCl/8 mM- Na_2HPO_4 /1.5 mM- KH_2PO_4 , pH 7.2).

H.p.l.c. analysis of lipids

Samples of lipids extracted from labelled cells were analysed on Spherisorb ODS 2 (5 μm particle size) with internal lipid standards as described previously (McIlhinney *et al.*, 1985).

Gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed on gradient gels of 7.5–15% acrylamide in the discontinuous buffer system of Laemmli (1970). Samples were prepared for electrophoresis as described previously (McIlhinney *et al.*, 1985) and, unless otherwise stated, all tracks on gels were loaded with equivalent amounts of protein (100 μg) assayed by using the Bio-Rad dye-binding assay with bovine serum albumin as a protein standard. The gels were then impregnated with 2,5-diphenyloxazole (Bonner & Laskey, 1974), dried and exposed with Kodak XS-1 X-ray film at –70 °C.

Subcellular fractionation

Cells (75 cm² flasks) labelled with fatty acids were washed with TNM buffer, scraped from the culture dishes and centrifuged (300 g_{av} for 10 min) to give a cell pellet. The cells were then resuspended in 5 mM-Tris/HCl buffer, pH 7.5 (3 ml), with vigorous pipetting to break up the cell pellet. The suspension was then sonicated, by three 10 s cycles at setting 2 on a Microson Ultrasonic Cell Disruptor. All of these manipulations were performed at 4 °C. The cell lysate was centrifuged at 400 g for 10 min to remove large cell debris, and then at 100000 g_{av} for 60 min to provide a crude membrane fraction. The supernatant from the high-speed spin was concentrated by adding 5 vol. of cold acetone and leaving at –20 °C for 24 h. The precipitate was collected by centrifugation and contained the cytosol proteins. The crude membrane fraction was treated with TNM buffer (1 ml) containing Triton X-100 (1%, v/v) and 1 mM-phenylmethanesulphonyl fluoride for 15 min at 4 °C. The resulting solution was centrifuged at 40000 g_{av} for 30 min, and the solubilized membrane proteins were recovered from the supernatant by precipitation with 5

Fig. 1. SDS/polyacrylamide-gel analysis of different subcellular fractions of LICR-LON HN5 cells labelled with palmitic acid (lanes a, b, c, e and g) and myristic acid (lanes d, f and h)

Cells were labelled and fractionated as described in the Materials and methods section. The pattern of total labelled proteins (lanes c and d) was compared with those of the cytosol (lanes a, e and f) and membrane fraction (lanes b, g and h) on a linear 7.5–15% polyacrylamide gradient gel. Lanes c, d, e, f and h were loaded with 100 μ g of protein, whereas lane g contained only 70 μ g. To compensate for this the gel was exposed for twice as long (8 weeks) and lane b shows the resultant autoradiograph; for comparison the longer exposure of the palmitate-labelled-cytosol lane e has also been included (lane a). The top and bottom of the separating gel are indicated by the double arrows, and the M_r markers are myosin (200000), phosphorylase *b* (93000), bovine serum albumin (69000), ovalbumin (46000), carbonic anhydrase (29000) and lysozyme (14500).

vol. of cold acetone as described above. This treatment was necessary to remove the large amounts of non-protein-bound fatty acids in the membranes, which tended to obscure the lower- M_r proteins on the SDS gels.

Immunoprecipitation of antigens

Cells (25 cm² flasks) labelled with [³⁵S]methionine were lysed in 1 ml of TNM buffer supplemented with Triton X-100 and phenylmethanesulphonyl fluoride as described above, and also containing SDS (0.1%), for 5 min at 4 °C. The lysate was centrifuged at 20000 $g_{av.}$ for 15 min and sodium deoxycholate added to give a final concentration of 0.5% (w/v). Samples (0.5 ml) of the supernatant were then immunoprecipitated for EGF receptor with the monoclonal antibody EGF-R1 coupled to Sepharose (Gusterson *et al.*, 1985). Equal portions of the lysate were incubated with the monoclonal antibody BT12/32 (10 μ g) to transferrin receptor (Trowbridge & Omary, 1981) for 2 h at 4 °C with rotation, after which 50 μ l of a 1:1 (v/v) suspension of Protein A–Sepharose (Sigma) in TNM was added and rotated for a further 2 h. The Protein A–Sepharose beads were then washed three times with TNM buffer containing Triton X-100 (1%, v/v), and the bound material was released by heating with SDS/polyacrylamide-gel sample buffer (Gusterson *et al.*, 1985). The eluted proteins were analysed on SDS/10%-polyacrylamide gels.

Detergent-binding studies

Triton X-114 was purified as described by Bordier (1981), and was used at a final concentration of 1% (w/v) in 1 ml of TNM buffer to lyse cells (25 cm² flasks) labelled with either myristic acid or palmitic acid. The cell lysates were centrifuged (20000 $g_{av.}$) for 30 min and, after removal of samples for analysis of total labelled proteins, the lysates were warmed to 30 °C and the phase separation was performed as described by Bordier (1981). The proteins from the pooled lower and upper phases were concentrated by acetone precipitation, and analysed by electrophoresis on SDS/polyacrylamide gels. All of the lower-phase proteins were analysed, but only a quarter of the upper-phase proteins, since these were the majority of the recovered proteins and would have overloaded the gels.

RESULTS

Cellular location of labelled proteins

In order to ascertain whether the cell proteins with attached lipids were membrane proteins, LICR-LON HN5 cells were labelled with either [³H]palmitic acid or [³H]myristic acid, then lysed and subjected to subcellular fractionation to provide a crude total membrane preparation and a cytosol fraction. These were then analysed by SDS/polyacrylamide-gel electrophoresis as

Fig. 2. SDS/polyacrylamide-gel analysis of (lanes a and b) the total cell proteins of LICR-LON HN5 cells, (lanes c and d) the proteins remaining in the upper Triton X-114 phase and (lanes e and f) those partitioning into the lower Triton X-114 phase

Cells were labelled with either palmitic acid (lanes b, d and f) or myristic acid (lanes a, c and e) and subjected to extraction with Triton X-114 as described in the Materials and methods section; the extracted proteins were then analysed on a linear 7.5–15% -polyacrylamide gradient gel, with 100 μ g of protein per track. The top and bottom of the separating gel are indicated by the double arrows, and the M_r markers are myosin (200000), phosphorylase *b* (93000), bovine serum albumin (69000), ovalbumin (46000), carbonic anhydrase (29000) and lysozyme (14500).

shown in Fig. 1. As reported previously (McIlhinney *et al.*, 1985), the two fatty acids label different populations of proteins (Fig. 1, lanes c and d). The proteins labelled by palmitic acid are clearly predominantly membrane proteins (Fig. 1, lanes b, c and g), with few bands present in the cytosol (Fig. 1, lane e) even after prolonged exposure of the autoradiograph (Fig. 1, lane a). The myristate-labelled proteins have a more complicated distribution, with some bands clearly enriched or present only in the cytosol (e.g. the bands indicated by the dots in Fig. 1, lane f) whereas others are found predominantly in the membrane fraction (e.g. the broad set of bands running at $M_r > 46000$ in Fig. 1, lane h). Other protein bands appear to be present in both fractions.

Detergent-binding properties of the lipid-labelled proteins

Given the differences in the cellular location of the proteins labelled by the two fatty acids, their ability to bind detergent was investigated with the use of the detergent Triton X-114. This non-ionic detergent has the property that at 4 °C it is soluble in aqueous buffers, but

Fig. 3. SDS/polyacrylamide-gel analysis of immunoprecipitates obtained with an anti-(EGF receptor) antibody (lane a) and an anti-(transferrin receptor) antibody (lanes b and c)

Cells (LICR-LON HN5) were labelled with either [35 S]methionine (lanes a and b) or [3 H]palmitic acid (lane c) and immunoprecipitation was performed as described in the Materials and methods section. The precipitated proteins were analysed on either SDS/7.5% -polyacrylamide (lane a) or SDS/10% -polyacrylamide (lanes b and c) gels. The EGF receptor gel (lane a) was run separately from that for the transferrin receptor (lanes b and c) but tracks b and c were run on the same gel, although the palmitate-labelled track (lane c) was exposed for 3 months, compared with the 16 h for the 35 S-labelled tracks. The M_r markers are myosin (200000), phosphorylase *b* (93000) and bovine serum albumin (69000).

Table 1. Composition of the major fatty acids from [3 H]acetate-labelled LICR-LON HN5 and LICR-LON HN6 cells

Cells were labelled with [3 H]acetate and the total fatty acids recovered as described in the Materials and methods section. The fatty acids were analysed by h.p.l.c., with unlabelled fatty acids as internal standards, and the results were calculated allowing for the differences in the relative contribution of acetate to each fatty acid. Each result represents the mean $\pm$ S.E.M. for four separate experiments on each cell line.

Cell line	% of total lipid as		
	Myristic acid	Palmitic acid	Stearic acid
LICR-LON HN5	9.9 $\pm$ 0.46	61.2 $\pm$ 1.6	17.9 $\pm$ 0.7
LICR-LON HN6	7.9 $\pm$ 0.46	60.2 $\pm$ 2.1	23.2 $\pm$ 2.2

at 30 °C it undergoes a phase separation and can be removed from solution by centrifugation. Integral membrane proteins and proteins that bind detergent will be found in the detergent phase at 30 °C, whereas proteins that do not bind detergent will remain in the aqueous phase (Bordier, 1981). LICR-LON HN5 cells were labelled with either myristic acid or palmitic acid, and the cell proteins were solubilized in Triton X-114 at 4 °C. A sample of the total cell lysate was kept, and then the lysate was allowed to warm to 30 °C. The resulting solution was centrifuged (50000 *g*-min) to separate the two phases, which were washed as described by Bordier (1981). The upper and lower phases were pooled separately, and the proteins in each were recovered by acetone precipitation. Samples from each were analysed by electrophoresis on SDS/polyacrylamide gels, and the results indicate that most of the palmitate-labelled

Fig. 4. H.p.l.c. analysis of the lipids released from [^3H]acetate-labelled LICR-LON HN5 cells by alkali hydrolysis

Cells were labelled with [^3H]acetate, and their proteins were extracted and washed extensively with organic solvents before treatment with KOH in methanol as described in the Materials and methods section. The lipids released were analysed by h.p.l.c., with the internal standards methyl myristate (mm), methyl palmitate (mp) and methyl stearate (ms) running as shown.

Fig. 5. H.p.l.c. of lipids released from ^3H -labelled LICR-LON HN5 cells by acid hydrolysis

After alkali hydrolysis of proteins labelled with [^3H]acetate, the cell proteins were subjected to acid hydrolysis in methanolic HCl as described in the Materials and methods section. The lipids released were analysed by h.p.l.c., with the internal standards myristic acid (m), methyl myristate (mm), methyl palmitate (mp) and methyl stearate (ms) running as shown.

proteins behave as hydrophobic proteins, partitioning into the lower phase with the detergent (Fig. 2, lanes b, d and e), the major exception being a high- M_r component (Fig. 2, lane d). However, many of the proteins labelled with myristic acid remain in the aqueous phase, with only a few (Fig. 2, lane e: indicated by dots) showing any enrichment in the lower phase. Although some of the myristate-labelled proteins appear to be present equally in both compartments, the fraction of these proteins present in the lower phase represents less than 25% of the total myristic acid label, since only one-quarter of the total cell lysate was loaded, compared with all of the lower phase.

Experiments to test the attachment of lipid to EGF receptor

LICR-LON HN5 cells have a very high content of EGF receptor (approx. 10^7 sites/cell) (Cowley *et al.*, 1986). The cell line offers therefore a unique system to determine whether the EGF receptor carries covalently attached lipid. In order to test this, cells were labelled with myristic acid or palmitic acid and parallel cultures were labelled with [^{35}S]methionine. The cells were lysed, the lysates immunoprecipitated for EGF receptor, and the precipitates analysed by SDS/polyacrylamide-gel electrophoresis. Although precipitates from [^{35}S]methionine-labelled cells could readily be shown to contain the receptor (Fig. 3, lane a) despite prolonged autoradiography no detectable lipid label could be found in the EGF receptor from cells labelled with either myristic acid or palmitic acid (results not shown). That this failure to demonstrate the incorporation of lipid was not due to an inadequate incorporation of label was shown, for palmitic acid at least, by our ability to immunoprecipitate the transferrin receptor from the same lysates as were

used for the EGF receptor experiments (Fig. 3, lanes b and c). In none of these experiments could we label the transferrin receptor with myristic acid.

Lipids attached to protein labelled by [^3H]acetate

Incubation of the two cell lines with [^3H]acetate for 48 h resulted in the labelling of both fatty acids and proteins. Routinely myristic acid, palmitic acid and stearic acid made up 90% of the fatty acids released by alkali hydrolysis of the organic-solvent-extractable material from both cell lines, the remainder containing unsaturated and longer-chain fatty acids. The relative proportions of these major fatty acids are given in Table 1, which shows that palmitic acid is the major component of the fatty acid pool in these cells. Since the relative contributions of these fatty acids to cell pools appear to be similar in the two cell lines and, as later experiments were to demonstrate, the results on the types and amounts of labelled fatty acid released from cell proteins from the two cell lines were very similar, the results from both cell lines have been pooled for quantitative purposes in the following experiments.

Alkali treatment of the proteins of these cells, after their exhaustive organic-solvent extraction, released considerable quantities of both palmitic acid and stearic acid, as well as small amounts of myristic acid (Fig. 4). Subsequent acid treatment of the alkali-resistant label attached to these cell proteins released further myristic acid, palmitic acid and stearic acid (Fig. 5), and similar results were obtained for the cell line LICR-LON HN6. Since further hydrolysis did not release further labelled lipid, these two hydrolyses served to remove all of the detectable fatty acid attached to the cell proteins. Over six experiments the lipid attached to protein averaged $2.1 \pm 0.5\%$ of the total extractable cell lipid.

Table 2. Composition of the fatty acids released from cell proteins of LICR-LON HN5 and LICR-LON HN6 cells by sequential alkali and acid hydrolyses

The cells were labelled with [^3H]acetate and the proteins were extracted and subjected to sequential alkali and acid hydrolyses as described in the Materials and methods section. The released fatty acids were analysed by h.p.l.c. (e.g. Figs. 4 and 5), and the amount of each fatty acid was calculated as a percentage of the total fatty acid released from cell protein, taking into account the relative contribution of acetate to each fatty acid. The results (means $\pm$ S.E.M.) from a total of six separate experiments, three on each cell line, have been pooled in this Table.

Hydrolysis	% of total fatty acid released as			
	Myristic acid	Palmitic acid	Stearic acid	Total
KOH	4.3 $\pm$ 1.4	44.5 $\pm$ 3.8	35.8 $\pm$ 1.9	84.6 $\pm$ 4.3
HCl	4.2 $\pm$ 1.5	6.4 $\pm$ 1.9	4.8 $\pm$ 1.61	15.4 $\pm$ 4.3

Analysis of the radioactively labelled fatty acids released from the cell proteins by each hydrolysis, taking into account the different relative contributions made to each by acetate, yielded the results shown in Table 2. This shows that the major fatty acids attached to protein are palmitic acid and stearic acid, which account for 90% of the lipid covalently bound to protein. Of the bound lipid 84.6% can be released by alkali treatment, the remainder needing acid hydrolysis to remove it. Relatively small amounts of palmitic acid and stearic acid are released by the acid treatment, compared with those released by alkali treatment, but proportionally more myristic acid is released by the acid treatment.

DISCUSSION

The results presented above on the cellular localization of the cell proteins with attached fatty acid are in agreement with those reported elsewhere (Magee & Courtneidge, 1985; Olson *et al.*, 1985) with palmitate-labelled proteins, as shown for rhodopsin (O'Brien & Zatz, 1983), histocompatibility antigens (Kaufman *et al.*, 1984) and transferrin receptor (Omary & Trowbridge, 1981), being predominantly membrane-associated. Some of the better-characterized myristate-labelled proteins are also associated with cell membranes, for example p60^{src} (Garber *et al.*, 1983; Sefton *et al.*, 1982) and NADH-cytochrome b_5 reductase (Ozols *et al.*, 1984), but others, such as calcineurin B (Aitken *et al.*, 1982) and cardiac-muscle cyclic-AMP-dependent protein kinase (Carr *et al.*, 1982), are found in the cytosol. The observations described here and by Olson *et al.* (1985) of myristate-labelled proteins present in both the cell cytosol and membranes could indicate that these are proteins that move between these two cellular compartments, with myristic acid facilitating their interaction with cell membranes. Such a role for myristic acid is suggested by the results obtained by Pellman *et al.* (1985), showing that the attachment of myristic acid to otherwise soluble proteins can direct them to the cell membrane, and is consistent with the observation that a point mutation in the codon for the *N*-terminal glycine of p60^{src}, converting it into alanine, is sufficient to

prevent the attachment of myristic acid and the stable association of p60^{src} with the cell membrane (Kamps *et al.*, 1985).

As might be expected from their close membrane involvement, the palmitate-labelled proteins behaved as integral membrane proteins in the detergent Triton X-114. The myristate-labelled proteins, however, behaved predominantly as soluble proteins, remaining in the upper aqueous phase after phase separation of the detergent. These results indicate a means by which myristate-labelled proteins might be solubilized and then freed of detergent in order to facilitate their purification.

Despite the presence on LICR-LON HN5 cells of 10^7 EGF-receptor sites/cell (Cowley *et al.*, 1986), we were unable to detect attachment of myristic acid or palmitic acid to this protein, even though we were able to show attachment of palmitic acid to transferrin receptor as described by Omary & Trowbridge (1981). Similar results were reported by Magee & Courtneidge (1985), with the cell line A431; however, since LICR-LON HN5 cells have 10 times the receptor density of A431 cells, the results described here make it unlikely that EGF receptor has attached lipid even on a subpopulation of molecules.

The major conclusion to be drawn from our use of acetate to label the lipids attached to cell proteins is that only myristic acid, palmitic acid and stearic acid are found attached to protein. Although this was suggested by previous studies using radioactive palmitic acid or myristic acid to label cells (McIlhinney *et al.*, 1985; Magee & Courtneidge, 1985; Olson *et al.*, 1985), the use of radioactive acetate to label all cell lipids reinforces the conclusion that only these three unsaturated fatty acids, of chain length C_{14} – C_{18} , are covalently attached to cell proteins.

The predominant fatty acids linked to the proteins by bonds susceptible to alkali treatment were palmitic acid and stearic acid. Generally fatty acids released from protein by such mild hydrolyses have been regarded as linked to the protein by an ester bond, probably as a thioester with the side chain of cysteine (Magee *et al.*, 1984; Kaufman *et al.*, 1984). The small amounts of myristic acid seen attached via an ester linkage could reflect the degree to which the acylation enzyme(s) responsible for the attachment of palmitic acid and stearic acid can utilize myristic acid, since labelling with either acetate or myristic acid yields a small amount of ester-linked myristic acid (McIlhinney *et al.*, 1985; Olson *et al.*, 1985).

Myristic acid formed a greater proportion (30%) of the released fatty acids after acid hydrolysis. This result is consistent with those obtained from cells labelled with myristic acid, which have shown that most of this fatty acid is only released by acid hydrolysis of cell proteins (McIlhinney *et al.*, 1985; Olson *et al.*, 1985). Characterization of the bond linking myristic acid to a number of cell proteins has shown that it is usually attached via an amide linkage involving the *N*-terminal amino acid of the protein, which is often glycine (Carr *et al.*, 1982; Aitken *et al.*, 1982; Henderson *et al.*, 1983). The small amounts of amide-bound palmitic acid and stearic acid found in the acid hydrolyses of cell proteins in our study may reflect the accumulation of errors in the specificity of the enzyme(s) normally responsible for the addition of myristic acid over the long incubation times used in these experiments.

The results described above are in good agreement with those obtained in the only other study to use radioactive acetate to label the fatty acids endogenously attached to cell proteins (Towler & Glaser, 1986). The principal differences in the results obtained in the two investigations concern the amount of palmitic acid attached to protein by an amide bond, and the amount of ester-linked stearic acid contributing to the total protein-bound lipid, Towler & Glaser (1986) reporting more of the former and less of the latter than were found in our study. However, it seems likely that these discrepancies may be accounted for by the use of different cell lines and labelling protocols in the two studies.

In conclusion, the results presented here show that the fatty acid acylation of cell proteins is a widespread phenomenon, and that myristic acid, palmitic acid and stearic acid are the major fatty acids found attached to cell proteins. The last two fatty acids are attached to predominantly membrane proteins by enzyme systems and bonds different from those that attach myristic acid to cell proteins, whereas the proteins with attached myristic acid are found more generally in cells and are not confined to the cell membranes.

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