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Two classes of fatty acid acylated proteins exist in eukaryotic cells

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Labelling of cultured cells with [^{3}H]palmitic and [^{3}H]myristic acids demonstrates that each of these fatty acids modifies a substantially different subset of cellular proteins. Hydroxylamine treatment can be used to differentiate sensitive thioester linkages to palmitate from insensitive amide linkages to myristate. Several palmitoylated proteins are surface-oriented glycoprotiens while all of the myristylated proteins appear to be internal. Myristate addition is much more tightly coupled to protein synthesis than palmitoylation, which is able to continue at a reduced level even in the prolonged absence of protein synthesis. Acyl proteins patterns were affected both qualitatively and quantitatively by transformation and growth status. The preferential addition of palmitate to the transferrin receptor and myristate to pp60^{src}, and the absence of these modifications from several other proteins is reported. We propose a nomenclature for fatty acyl proteins based on these observations.

Key words: fatty acids/hydroxylamine/protein acylation

Introduction

In recent years much attention has been focussed on the post-translational addition of long chain fatty acids to eukaryotic proteins (reviewed in Magee and Schlesinger, 1982; Schmidt, 1983). The majority of the studies reported have concentrated on viral proteins as models of normal cell glycoproteins (Schmidt *et al.*, 1979; Schmidt and Schlesinger, 1980; Schmidt, 1982) or plasma membrane-associated tumour virus proteins (Sefton *et al.*, 1982; Henderson *et al.*, 1983; Klockmann and Deppert, 1983).

A growing number of investigators have observed fatty acid acylation of uninfected cell proteins (Schlesinger *et al.*, 1980; Keenan *et al.*, 1982) in particular the transferrin receptor, histocompatibility antigens, cAMP-dependent protein kinase and calcineurin B (Omary and Trowbridge, 1981; Kaufman *et al.*, 1984; Carr *et al.*, 1982; Aitken *et al.*, 1982). The phenomenon of fatty acid acylation of proteins has been detected in such diverse organisms as bacteria (reviewed in Schlesinger, 1981), yeast (Wen and Schlesinger, 1984), *Dictyostelium* (Stadler *et al.*, 1984), trypanosomes (Ferguson and Cross, 1984) and sea urchin (Bolanowski *et al.*, 1984). The functions of protein-bound fatty acyl moieties are as yet ill-defined but they have been implicated in the mode of association of transforming gene products with the inner face of the plasma membrane (Sefton *et al.*, 1982; Garber *et al.*, 1983), in modulating the protease-sensitivity of gastric mucus (Slomiany *et al.*, 1984), and as markers during development of sea urchin embryos (Bolanowski *et al.*, 1984).

Two types of fatty acid-protein linkage have been identified: hydroxylamine-sensitive thioester linkage to the side-chain of cysteine residues (Magee *et al.*, 1984; Kaufman *et al.*, 1984) and hydroxylamine-insensitive amide linkage to the amino-terminal residue of several proteins (Carr *et al.*, 1982; Aitken *et al.*, 1982; Henderson *et al.*, 1983). The former type of linkage appears to involve the most abundant cellular acyl chains, i.e., palmitate ($C_{16:0}$), stearate ($C_{18:0}$) and oleate ($C_{18:1}$) (Schmidt *et al.*, 1979), while the amide linkage is exclusively to myristate ($C_{14:0}$), a low abundance acyl chain.

We have undertaken a study of total protein acylation in cultured cells as a first step towards defining the properties of acyl proteins and their functional roles in membrane association and membrane traffic. Our data show that at least two classes

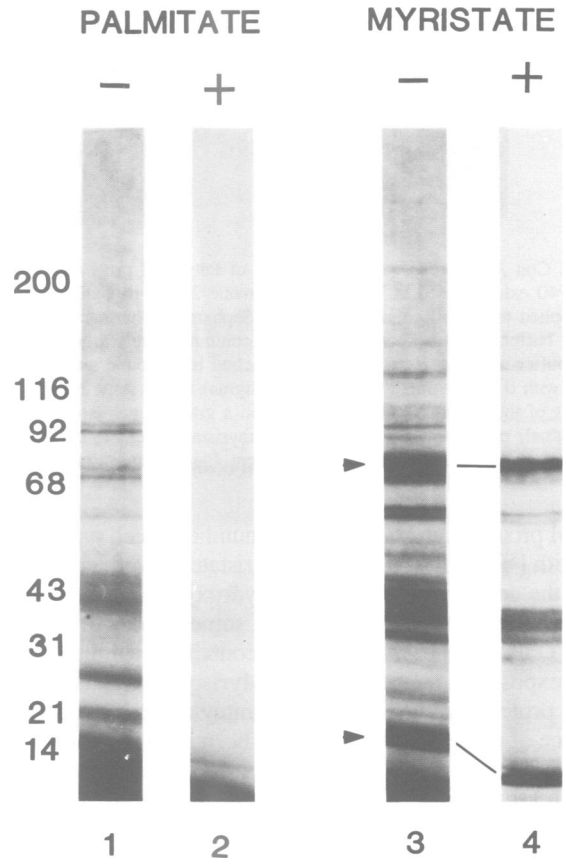

Fig. 1. Fatty acid labelling of chicken embryo fibroblasts. Newly confluent cultures of CEF in 35 mm dishes were labelled with tritiated palmitate **(lanes 1** and **2)** or myristate **(lanes 3** and **4)** for 13 h and extracted with 0.4 ml 0.5% NP40 − 20 mM Tris − 1 mM EDTA − 0.15 M NaCl − pH 7.5 (RIP buffer) at 0°C for 5 min. Lysates were cleared by brief centrifugation at 10 000 *g* for 2 min and electrophoresed in duplicate on a 5 − 15% linear gradient SDS-polyacrylamide gel. One half of the gel was treated with (+, **lanes 2** and **4)** or without (−, **lanes 1** and **3)** hydroxylamine as described in Materials and methods followed by fluorography for 10 days on pre-flashed X-Omat G. Arrowheads show the two major myristylated proteins. Mol. wt. markers indicated on the left were: myosin (200 kd); β-galactosidase (116 kd); phosphorylase (92 kd); bovine serum albumin (68 kd); ovalbumin (43 kd); carbonic anhydrase (31 kd); soybean trypsin inhibitor (21 kd); lysozyme (14 kd).

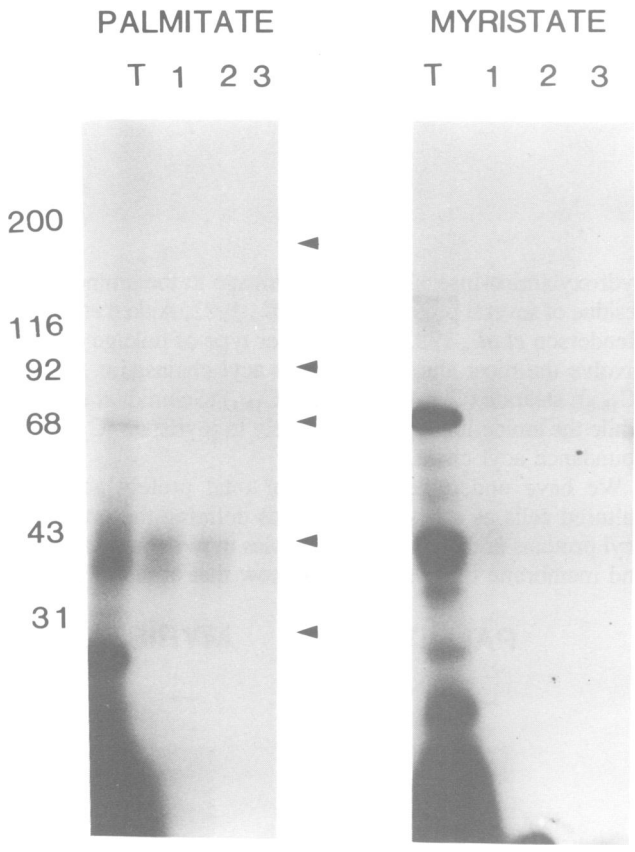

Fig. 2. Con A-Sepharose chromatography of fatty acyl proteins. 320 μl of the NP40 extracts used in Figure 1 were made 2 mM in $CaCl_2$ and $MnCl_2$ and applied to 0.3 ml columns of Con A-Sepharose (Pharmacia) equilibrated in RIP buffer with Ca^{2+} and Mn^{2+}. The columns were washed with the same buffer until the eluted counts approached background and were then eluted with 0.1 M α-methyl mannoside (Sigma) in the same buffer. Equal aliquots of the eluted fractions were run on a gradient gel as in Figure 1. **Left panel,** palmitate label; **right panel,** myristate label. **T**, unbound material; **1,2,3**, fractions eluted with hapten. Fluorography was for 7 days on pre-flashed XOmat G film.

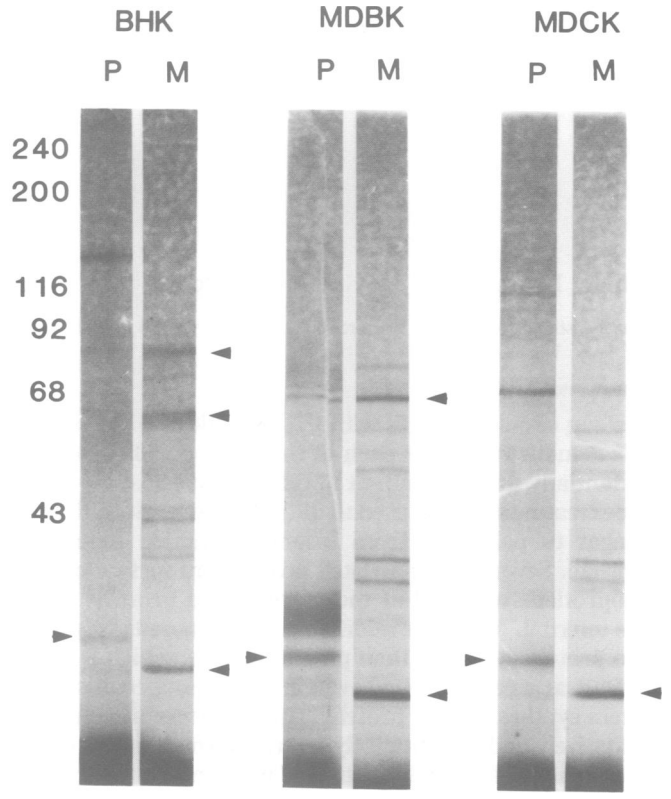

Fig. 3. Fatty acyl proteins of cultured mammalian cell lines. Newly confluent cultures of the indicated cell lines in 35 mm dishes were labelled for 4.5 h with [^{3}H]palmitate (P) or [^{3}H]myristate (M), extracted with gel loading buffer and analysed as described in Figure 1. Rightward-pointing arrowheads show the major palmitoylated protein, leftward-pointing arrowheads indicate the major myristylated proteins. Mol. wt. markers are as for Figure 1 except for fibronectin (240 kd). Fluorography was for 8 days on pre-flashed XAR-5 film.

of acyl proteins can be defined in a number of cell types by labelling with [^{3}H]palmitate and [^{3}H]myristate as well as by sensitivity of the acyl-protein linkage to hydroxylamine. Most of these proteins are membrane-bound and some appear to be glycosylated. Only some of those proteins containing palmitic acid seem to be exposed at the cell surface. Myristylation is tightly coupled to protein synthesis while palmitoylation can occur in the absence of protein synthesis as has been reported for transferrin receptor (Omary and Trowbridge, 1981). The two types of acyl protein are epitomised by the transferrin receptor for palmitoyl proteins and the viral and cellular forms of tyrosine kinase $pp60^{src}$ for myristoyl proteins. In addition, the level of expression of some acyl proteins may be related to cell growth status or transformation. Neither fatty acid could be detected on the epidermal growth factor receptor, middle T antigen of polyoma virus or the $pp60^{v\text{-}src}$ substrate pp 36.

Results

As previously described (Schlesinger *et al.*, 1980) cultured chicken embryo fibroblasts contain a number of proteins which label specifically with [^{3}H]palmitic acid, notably a band of 24 kd (Figure 1, lane 1). Treatment with hydroxylamine under mild conditions releases most of this label (Figure 1, lane 2) which has been proposed to be due to linkage of the fatty acid *via* a thioester to cysteine side chains (Magee *et al.*, 1984). In contrast, parallel cultures labelled with [^{3}H]myristic acid show a substantially different pattern of acylated proteins (Figure 1, lane 3). In particular two very prominent bands of 70 kd and 18 kd are observed (arrowheads in lanes 3 and 4). Most of these bands are resistant to hydroxylamine (Figure 1, lane 4), or alkaline methanol cleavage (not shown) consistent with linkage of fatty acid *via* amide bonds (Figure 1, lane 4). Neither of the fatty acid labelled patterns corresponded to the total protein distribution revealed by staining with Coomassie Blue or labelling with [^{35}S]methionine (not shown).

The glycoprotein nature of the acylated proteins was investigated by chromatography of [^{3}H]palmitate or [^{3}H]myristate-labelled extracts on Con A-Sepharose. A subset of the palmitoylated proteins bound and were specifically eluted with α-methyl mannoside, notably bands of 30 kd, 41 kd, 70 kd, 90 kd and 185 kd (arrowed in Figure 2A). Of the myristylated proteins only bands of 41 kd and 185 kd showed significant specific binding, and these appeared to be equivalent to the palmitoylated bands of the same mol. wts. (Figure 2B).

Labelling of other cultured cells

Established fibroblastic and epithelial cell lines of hamster (BHK), canine (MDCK) and bovine (MDBK) species were labelled with [^{3}H]palmitate and [^{3}H]myristate. Each cell type showed specific labelling with the two fatty acids and the patterns of acylated proteins were generally similar to those seen in chicken fibroblast cultures (Figure 3). The two major myristylated bands seemed

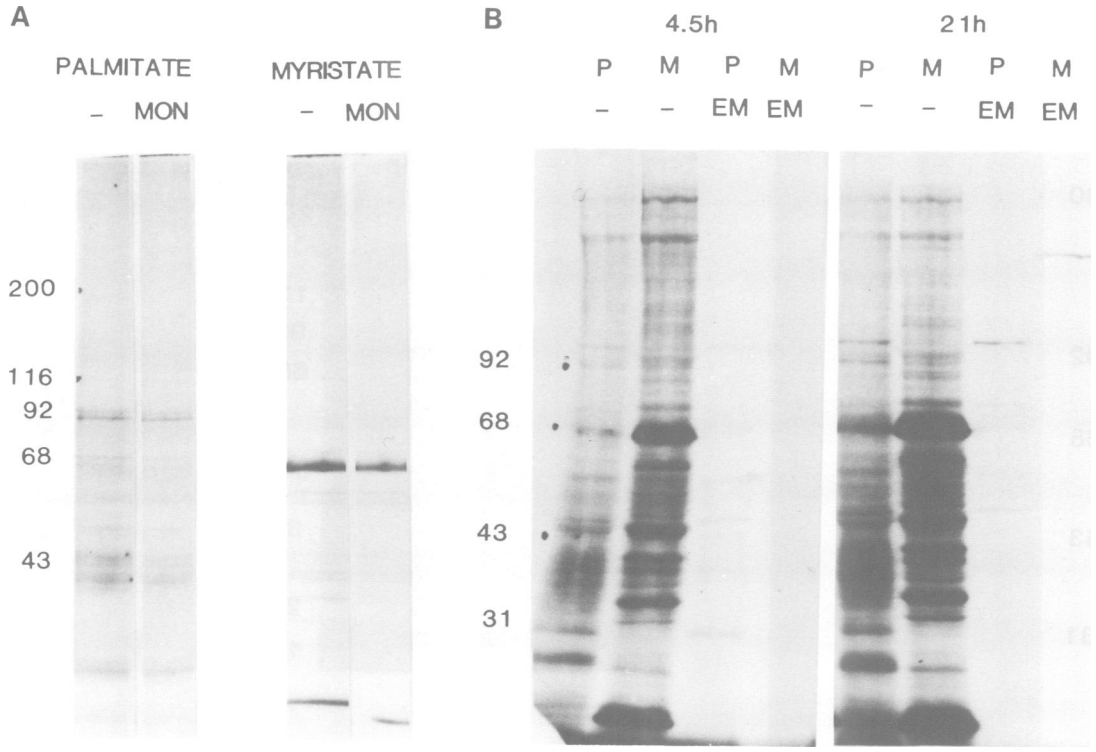

Fig. 4. Effect of inhibitors of membrane traffic and protein synthesis on fatty acid acylation. **Panel A:** newly confluent CEF in 35 mm dishes were pre-treated with 4 μM monensin (MON) (Sigma) for 2 h and then labelled for 4 h in the presence of the drug. Control cultures received no drug (−). Total cell lysates in gel loading buffer (0.2 ml) were prepared and analysed as before. **Panel B:** CEF were labelled for 4.5 h or 21 h with (EM) or without (−) treatment with 10 μM emetine for 2 h before and during the labelling period. P, [^{3}H]palmitate; M, [^{3}H]myristate. Fluorography: pre-flashed XAR-5 film for 6 days **(A)** or 10 days **(B)**.

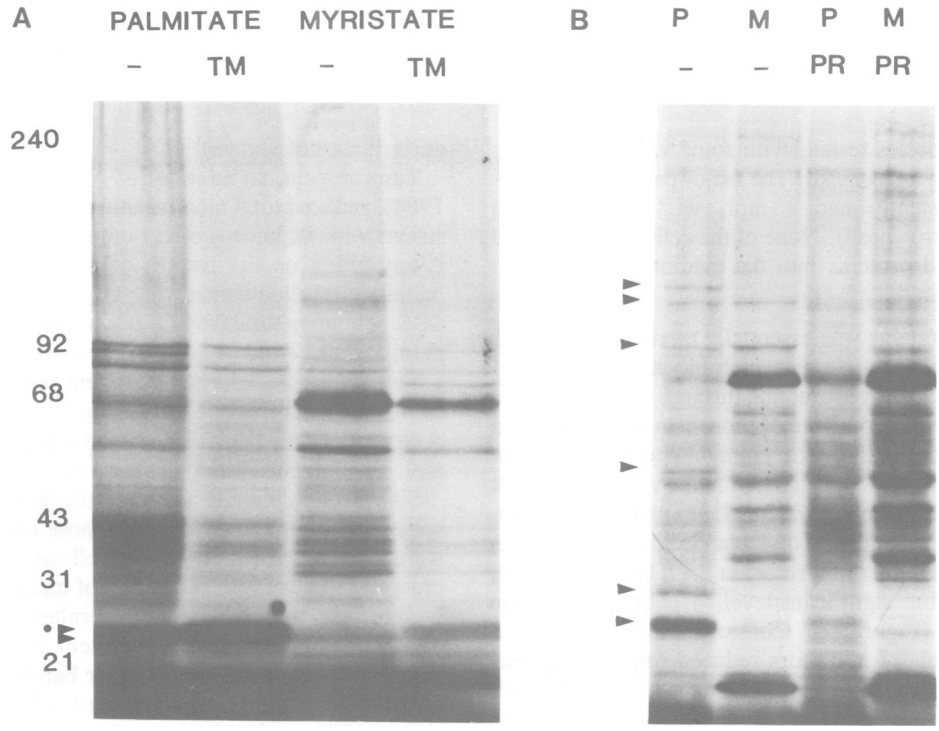

Fig. 5. Glycosylation and cell surface exposure of acyl proteins. **Panel A:** CEF in 35 mm dishes were pre-incubated for 3 h with (TM) or without (−) 0.5 μg/ml tunicamycin and labelled for 4 h with fatty acids. Total lysates in gel loading buffer were prepared and analysed on an SDS-polyacrylamide gel. Arrowheads show the major palmitoylated protein with (upper) or without (lower) tunicamycin. **Panel B:** CEF were labelled for 4.5 h with fatty acids, washed twice with warm phosphate-buffered saline and treated for 15 min at 37°C with 50 μg/ml trypsin plus 50 μg/ml chymotrypsin in serum-free medium, during which time the cells detached (PR). Control cultures were not treated (−). The cells were resuspended in 1 ml complete medium containing 10% serum, pelletted at 10 000 *g* for 1 min and lysed in 0.2 ml gel loading buffer. The arrowheads show the palmitoylated proteins lost after proteolysis. Exposure was on pre-flashed XAR-5 film for 22 days **(A)** or 10 days **(B)**.

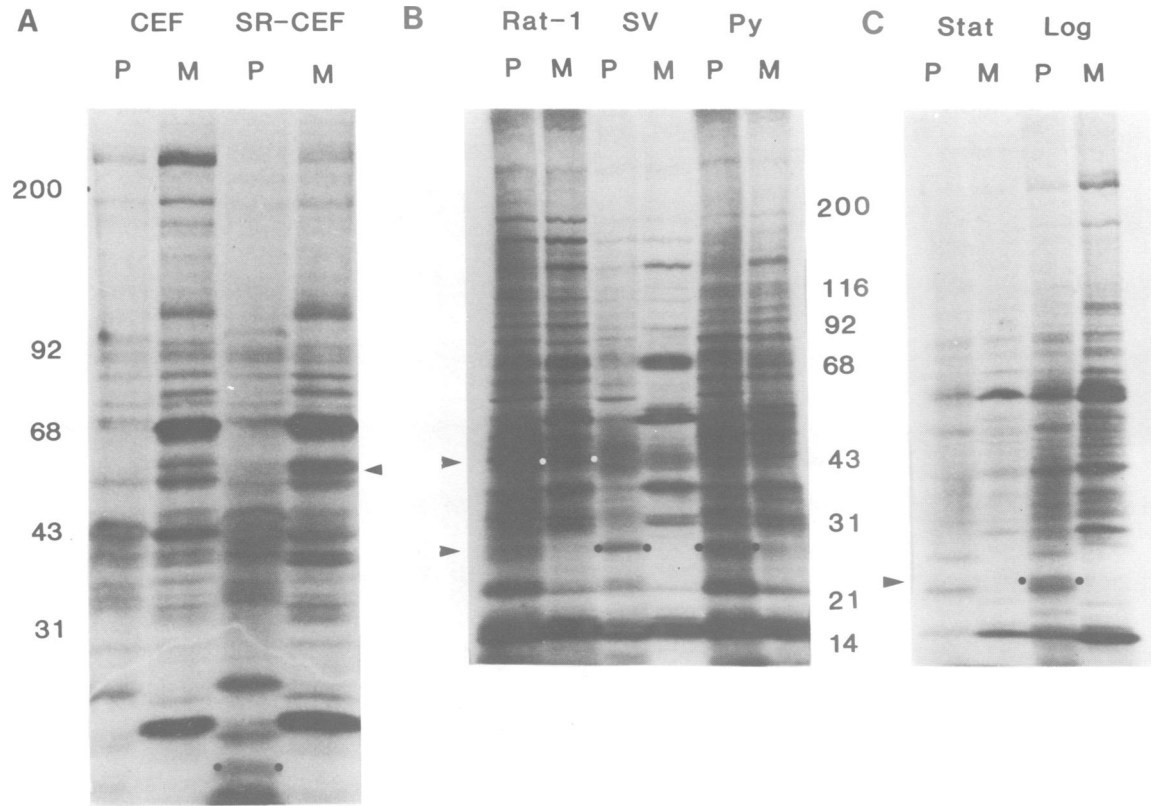

Fig. 6. Relationship of acylation to transformation and growth status. **Panel A:** confluent cultures of CEF or Rous sarcoma virus-transformed CEF (SR-CEF) were labelled for 4 h with fatty acids (P, palmitate; M, myristate), solubilised in gel loading buffer and analysed. The arrowhead shows the major myristylated band in the transformed cultures which corresponds to pp60$^{v\text{-}src}$. The transformation-related palmitoylated protein is also indicated. **Panel B:** Rat-1 cells or their transformants with SV40 virus (SV) or polyoma virus (Py) were labelled for 4 h with fatty acids, lysed in gel loading buffer and analysed. The transformation-related palmitoylated band is shown with the lower arrowhead. The upper arrowhead shows the myristylated band lost on SV40 transformation. **Panel C:** quiescent (Stat) and logarithmically growing (Log) cultures of CEF were labelled for 4 h with [^{3}H]palmitate (P) or [^{3}H]myristate (M), lysed in gel loading buffer and analysed on a gradient gel. The growth-related palmitoylated band is arrowed. Fluorography was for 5 – 12 days on pre-flashed XAR-5 film.

to be present in all species tested, with some variation in mol. wts. (leftward-pointing arrowhead). The major palmitoylated protein observed consistently had a mol. wt. around 24 kd (rightward-pointing arrowhead). None of the cells tested secreted any fatty acid-labelled proteins into the medium.

Effect of drugs on acylation

The pattern of acyl proteins was essentially unaffected by treatment with 4 μM monensin, a drug which blocks movement of secretory and membrane glycoproteins through the Golgi complex (Figure 4A). The temporal site of monensin action has been shown to be after the addition of palmitate to the envelope glycoproteins of Sindbis and vesicular stomatitis viruses (Schmidt and Schlesinger, 1980; Dunphy *et al.*, 1981; Quinn *et al.*, 1983).

Inhibitors of protein synthesis (cycloheximide and emetine) completely blocked addition of myristate in a 4 h labelling period, however, a reduced but significant level of palmitate addition was consistently seen (Figure 4B). Control experiments demonstrated that these levels of inhibitors reduced protein synthesis to $<2\%$ of controls (not shown). Labelling with palmitate for 21 h in the presence of emetine produced nearly control levels of fatty acid in bands of 46 kd, 50 kd, 55 kd, 73 kd and 100 kd (Figure 4B). This length of incorporation also demonstrated protein synthesis-independent myristylation of a few bands (35 kd, 68 kd, 180 kd). The combined effect of emetine and monensin was tested during short (5 h) and long (21 h) incorporations of label. Monensin did not reduce the level of emetine-resistant acylation (data not shown).

Tunicamycin, an inhibitor of N-linked glycosylation (Elbein, 1984), reduced total incorporation of myristate into protein consistent with its known effect on protein synthesis but had little if any effect on the pattern of myristylated proteins. Several palmitate-labelled bands were observed to change migration on SDS gels after tunicamycin treatment (Figure 5A). The major palmitoylated band of 24 kd shifted to a position consistent with a mol. wt. of 26 kd after treatment (arrowheads). The protein of 46 kd which had previously been observed to bind to Con A-Sepharose occasionally showed a slight increase in apparent mol. wt. but this was not observed consistently.

Since Con A binding and tunicamycin treatment suggested that some acyl proteins, particularly those containing palmitic acid, were glycosylated, we tested the cell surface accessibility of these proteins by proteolysis. Digestion of intact fatty acid-labelled cells with trypsin plus chymotrypsin resulted in almost complete loss of the major palmitoylated protein of 24 kd (arrowhead in Figure 5B) and some other minor bands (28 kd, 45 kd, 90 kd, 94 kd). The pattern of myristylated proteins was essentially unchanged except for the loss of a band of 90 kd.

Acylation of proteins in transformed cells

Transformation of chick embryo fibroblasts (CEF) with Schmidt-Ruppin A strain of Rous sarcoma virus resulted in quantitative changes in the level of acylation of many bands (Figure 6A), although the overall acyl protein pattern was generally unaffected.

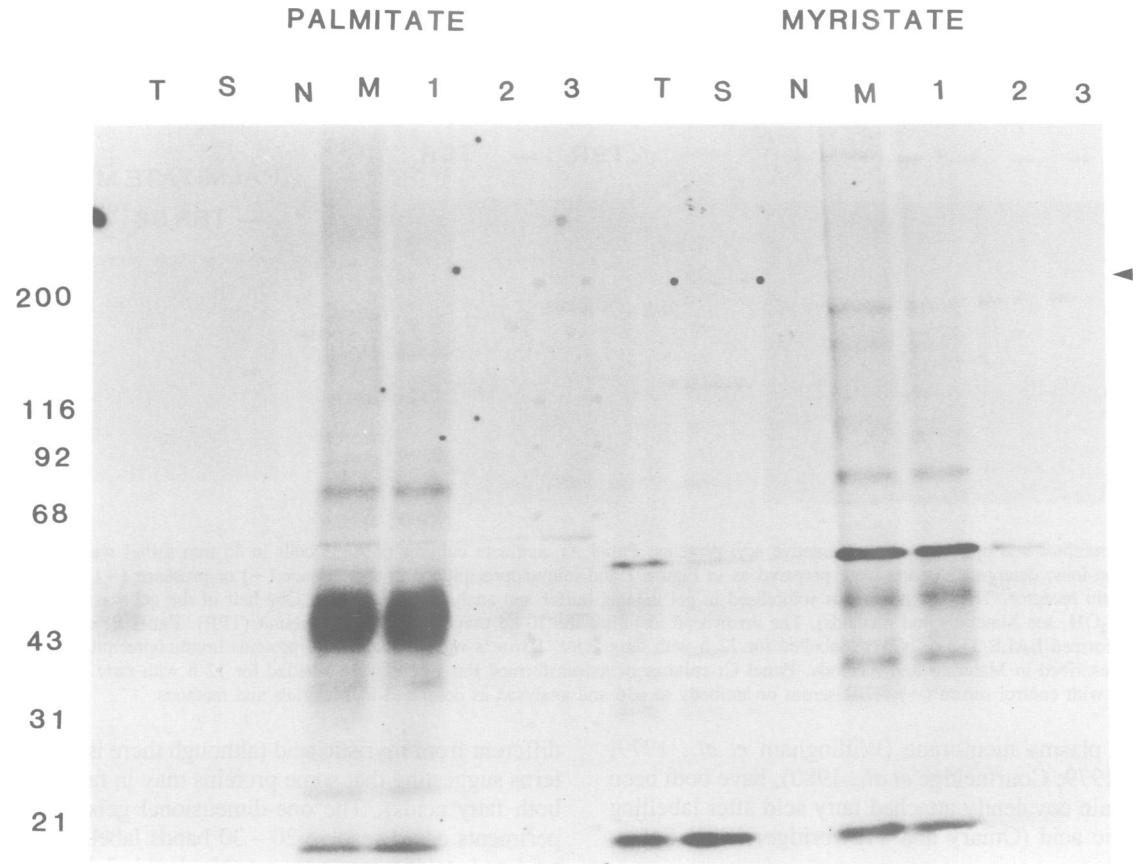

Fig. 7. Subcellular localisation of acyl proteins. Fatty acid labelled Rat-1 cells were fractionated as described in Materials and methods, and equal amounts of protein (50 μg) from each fraction were analysed on an SDS-polyacrylamide gradient gel. **T**, total extract; **S**, soluble fraction; **N**, detergent-washed nuclear fraction; **M**, total membranes; **1,2,3,** light, intermediate and dense membrane fractions from discontinuous sucrose gradient. Fluorography was for 21 days on pre-flashed XAR-5 film.

An exception to this was the appearance of a myristate-labelled band of 60 kd in the transformed cells (Figure 6A, arrowhead). This band was subsequently shown to be the transforming protein pp60$^{v\text{-}src}$ of Rous sarcoma virus (see below). Rat-1 cells and their transformants with SV40 and polyoma viruses were also examined. The patterns of acyl proteins were generally similar between the normal and transformed cells. SV40-transformed Rat-1 cells had acquired a major palmitoylated band of 27 kd (lower arrowhead, Figure 6B) and lost a myristylated band of 45 kd (upper arrowhead). Other changes were quantitative. Polyoma virus-transformed cells also had an increased level of the 27-kd palmitoylated band, and a reduced amount of a myristylated band at 72 kd. Both transformed cell lines had much reduced amounts of two bands (170 kd and 200 kd) which seemed to label equally efficiently with both fatty acids. It is not clear whether the changes in acyl protein pattern observed reflect changes in the amount of the proteins present or in their level of acylation.

To determine whether the changes in acylation patterns were due solely to transformation or were a consequence of the increased growth rate of the transformed cells, we labelled CEF during log phase growth and after reaching density arrest at confluence. The level of incorporation into stationary cultures was considerably lower but the most striking difference was the presence of a 25 kd palmitoylated band in the growing but not the stationary cultures (arrowhead, Figure 6C). This band may be related to the 27-kd band seen in the transformed Rat-1 cells.

Subcellular fractionation of acyl proteins in Rat-1 cells

Newly confluent cultures of Rat-1 cells were labelled with [^{3}H]myristate or [^{3}H]palmitate for 18 h and the cells were subjected to lysis followed by subcellular fractionation on discontinuous sucrose gradients according to the method of Courtneidge *et al.* (1983). The results show that the majority of acyl proteins are membrane bound and fractionate in discontinuous gradients with plasma membrane markers (Figure 7). No labelling was observed in the nuclear fraction. Some myristylated proteins were present in the cytosol, especially the major 18-kd band and a band of 200 kd (indicated). A band of 170 kd which labelled with both palmitate and myristate was enriched in denser membranes.

The possibility that some of the acylated bands were membrane-bound proteolipids (Schlesinger, 1981) was tested by partition in organic solvents using the system of Ames (1968). No palmitoylated or myristylated proteins partitioned specifically into the organic phase and in fact most of them precipitated at the interface. However, the 70-kd myristylated protein partitioned largely into the aqueous phase under these conditions (not shown).

Transferrin receptor and pp60^{src} epitomise the two classes of acyl proteins

To confirm the specificity of labelling with the two fatty acids, examples of known proteins were sought which epitomise each class of acyl protein. The transferrin receptor, a transmembrane surface-exposed glycoprotein and pp60$^{v\text{-}src}$, the transforming protein of Rous sarcoma virus which is associated with the in-

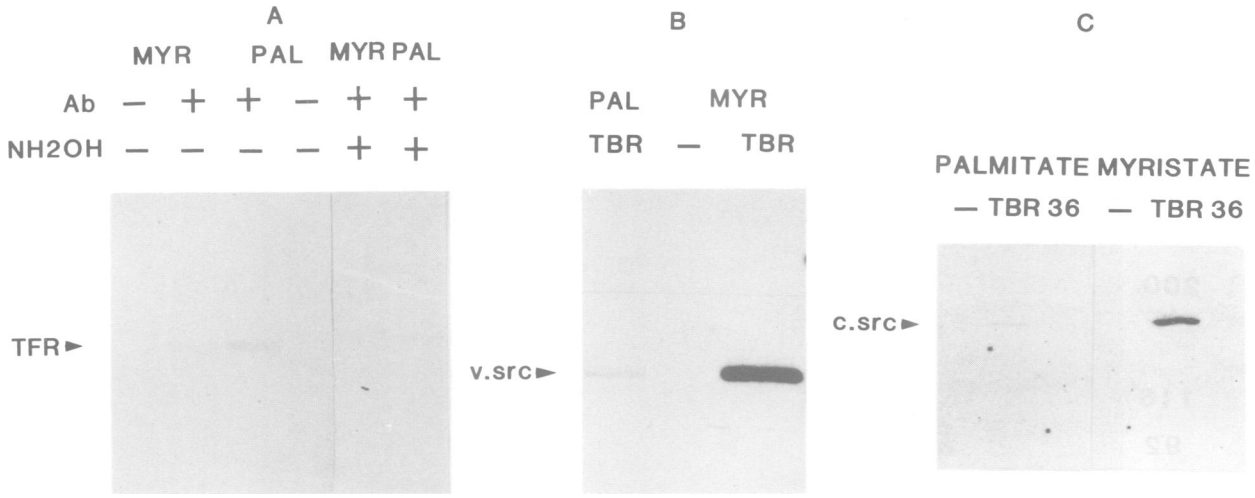

Fig. 8. Transferrin receptor and pp60^{src} are representative acyl proteins. **Panel A:** confluent cultures of A431 cells in 35 mm dishes were labelled for 4 h with fatty acids. Non-ionic detergent extracts were prepared as in Figure 1 and immunoprecipitated in the absence (−) or presence (+) of a monoclonal antibody to transferrin receptor. The precipitate was solubilised in gel loading buffer and analysed in duplicate. One half of the gel was treated with hydroxylamine (NH_2OH, see Materials and methods). The arrowhead identifies the 70-kd transferrin receptor subunit (TFR). **Panel B:** cultures of Rous sarcoma virus-transformed BALB/3T3 cells were labelled for 12 h with fatty acids. Extracts were prepared and proteins immunoprecipitated with control (−) or TBR serum as described in Materials and methods. **Panel C:** cultures of untransformed Rat-1 cells were labelled for 12 h with fatty acids, immunoprecipitated with control serum (−), TBR serum or antibody to p36 and analysed as described in Materials and methods.

ner face of the plasma membrane (Willingham *et al.*, 1979; Krueger *et al.*, 1979; Courtneidge *et al.*, 1980), have both been reported to contain covalently attached fatty acid after labelling with [^{3}H]palmitic acid (Omary and Trowbridge, 1981; Sefton *et al.*, 1982).

Human A431 tumour cells contain high levels of transferrin receptor (Hopkins and Trowbridge, 1983). These cells were labelled with each fatty acid, and non-ionic detergent extracts were prepared. Immunoprecipitation was performed with a monoclonal antibody to transferrin receptor (a kind gift of Dr. C. Schneider, ICRF, Mill Hill). Precipitates were solubilized in gel loading buffer containing reducing agent and run on an SDS gel in duplicate. Subsequently one half of the gel was treated with hydroxylamine and both halves were subjected to fluorography. Figure 8A shows that a band corresponding to the reduced mol. wt. of the transferrin receptor labels strongly with palmitate and weakly with myristate. Control precipitates did not contain this band. Treatment with hydroxylamine released the majority of label from the 70-kd band.

The converse was true when pp60$^{v\text{-}src}$ was immunoprecipitated from fatty acid-labelled cells (Figure 8B). Myristate labelling was much stronger than palmitate labelling and the fatty acid-protein linkage was insensitive to hydroxylamine cleavage (data not shown). The lack of absolute specificity of labelling could be due to some interconversion of the fatty acids (Berger and Schmidt, 1984) or to the tolerance of the putative acylating enzyme(s) to slight variations in chain length. Immunoprecipitation of pp60$^{c\text{-}src}$ from fatty acid-labelled Rat-1 cells confirmed that this protein also contained covalently attached myristic acid (Figure 8C). Attempts to demonstrate covalent attachment of fatty acids to human epidermal growth factor receptor, polyoma virus middle T antigen (data not shown) and the pp60^{src} substrate pp36 (Figure 8C) were unsuccessful.

Discussion

The data presented here demonstrate that fatty acid acylation of proteins is a common post-translational modification in eukaryotic cells. However, palmitic acid labels a subset of proteins distinctly different from myristic acid (although there is overlap in the patterns suggesting that some proteins may in fact be acylated with both fatty acids). The one-dimensional gels used in these experiments could resolve 20−30 bands labelled with each fatty acid and, taking into account this double labelling, this means that cells contain of the order of at least 40 acyl proteins. Most of the acyl proteins fractionate with plasma membrane as might be expected for proteins modified with a hydrophobic acyl chain. Nevertheless a number of myristylated proteins are present in significant amounts in the cytosol fraction. Recently two cytosolic proteins, the catalytic subunit of the cAMP-dependent protein kinase and calcineurin B (protein phosphatase-2B), have been shown to have amino termini blocked by myristic acid (Carr *et al.*, 1982; Aitken *et al.*, 1982). The amino-terminal myristylation of pp60^{src} seems to be required, but not sufficient, for the plasma-membrane localisation of the protein and for its transforming ability (Cross *et al.*, 1984; Buss *et al.*, 1984). It therefore appears that some myristyl proteins may be able to move between the cytosol and cell membranes, perhaps after a conformational change involving exposure of the acyl chain. On the other hand myristylation may have a role in the interactions of one protein with another or with a substrate.

In this report several palmitoyl proteins are shown to be glycosylated and exposed at the cell surface. However, many more of these proteins are unglycosylated and not externally exposed. Previously identified palmitoylated proteins have generally been cell surface glycoproteins (Schmidt *et al.*, 1979; Omary and Trowbridge, 1981; Kaufmann *et al.*, 1984) with the exception of p21^{ras} which is a cytoplasmically exposed membrane-associated protein (Sefton *et al.*, 1982). Since the sites of acylation of the viral membrane glycoproteins and the HLA protein appear to be cysteine residues located on the cytoplasmic side of the membrane (Magee *et al.*, 1984; Kaufmann *et al.*, 1984; Rose *et al.*, 1984) it is conceivable that the acylating enzyme has a cytoplasmically oriented active site, which would explain the ability to acylate p21^{ras} in a similar manner. In contrast, very few myristylated proteins have detectable carbohydrate nor are they susceptible to externally added protease. These proteins may

be almost exclusively cytoplasmically oriented as are all the examples known to date (Carr *et al.*, 1982; Aitken *et al.*, 1982; Schultz and Oroszlan, 1983; Buss *et al.*, 1984). Susceptibility to externally added protease is only an indication of cell surface exposure since some proteins are highly resistant to proteases whereas others have been shown to exist in large intracellular pools and may only spend a transient time on the cell surface (Steer and Ashwell, 1980; Wileman *et al.*, 1984). Therefore a more careful analysis of the acylation of proteins such as recycling receptors is required and this is currently under way in this laboratory. Thus far the transferrin receptor has been shown to contain covalently attached palmitate (Omary and Trowbridge, 1981; this study) while the epidermal growth factor receptor (see below) and the low-density lipoprotein receptor are unacylated (Brown *et al.*, 1983).

Addition of myristic acid is much more tightly coupled to protein synthesis than palmitate addition. This may reflect the temporal site of acylation which in the case of palmitate probably occurs 10 – 20 min after synthesis in the *cis*-Golgi (Schmidt and Schlesinger, 1980; Dunphy *et al.*, 1981; Quinn *et al.*, 1983). Myristate may be added immediately after or even during protein synthesis (Buss *et al.*, 1984). In agreement with this we have recently shown that blocking of protein synthesis with emetine immediately reduces incorporation of myristate into cell proteins whereas incorporation of palmitate continues for 5 – 10 min (A.I. Magee, unpublished observations). Some proteins, particularly those acylated with palmitate, seem to be able to label to normal levels in the absence of protein synthesis. This acylation is not blocked by monensin which inhibits recycling and export of proteins (Tartakoff, 1983).

Comparison of normal and virally transformed cells reveals a number of interesting effects on acylation. In general, the pattern of both palmitoylated and myristylated proteins is unchanged by transformation. In Rous sarcoma virus-transformed cells a major myristate-labelled band of 60 kd appeared which could be shown by immunoprecipitation to be pp60$^{v\text{-}src}$. The protein was labelled poorly by palmitic acid. The only other change was the increased amount of a palmitoylated band of 14 kd. Rat-1 cells transformed with either SV40 or polyoma viruses also showed increased palmitoylation of a band of 28 kd. A preliminary experiment with logarithmically growing and quiescent Rat-1 cells did not show any qualitative changes in the acyl protein pattern in this region of the gel. However, palmitate labelling of a band of 28 kd did seem to be growth related in chicken embryo cells. The nature of these differences is under investigation. It is not possible to tell at this stage whether these changes reflect a different level of acylation of these proteins or a change in their actual amount. In addition to pp60$^{v\text{-}src}$ the current study demonstrates that the cellular homologue pp60$^{c\text{-}src}$ is also preferentially labelled with myristic acid, an observation also made by Buss and Sefton (1985). This protein is also plasma-membrane associated (Courtneidge *et al.*, 1980). The membrane-associated pp60^{src} substrate pp36 (Courtneidge *et al.*, 1983) is not acylated nor is pp60$^{c\text{-}src}$-associated middle T antigen of polyoma virus (Courtneidge and Smith, 1983). Immunoprecipitation of transferrin receptor shows that it is preferentially acylated with palmitate. The palmitoylation of this protein has been previously demonstrated (Omary and Trowbridge, 1981). No acylation of epidermal growth factor receptor could be detected.

All of these observations demonstrate that neither fatty acid label is converted to amino acids under the labelling conditions used (Sefton *et al.*, 1982). The patterns of acyl proteins did not resemble each other for the most part, nor did they resemble the total protein pattern (for example, pp36 comprises 0.2% of total cell protein yet was not labelled with either palmitate or myristate). Loss of palmitate label on hydroxylamine treatment shows that the isotope has not been re-incorporated into amino acids. Consistent with specific labelling by each fatty acid we have illustrated the opposite labelling patterns of pp60^{src} (preferentially myristylated) and transferrin receptor (preferentially palmitoylated). These observations also argue against pool size effects prejudicing the assignment of a band as myristylated or palmitoylated. Despite preferential labelling with myristate or palmitate some labelling with the other fatty acid was usually observed. This could be due to limited specificity of the acylating enzymes or to a low level of interconversion of the fatty acids. Since Schmidt (1984) has shown that interconversion only occurs in the direction of chain elongation the former explanation seems more likely, at least for bands preferentially labelled with palmitate, although Buss and Sefton (1985) have recently challenged his observations. We propose that the pattern of labelling of proteins with both palmitate and myristate can form the basis of a classification of acyl proteins into one of the two groups. The acyl group modification could be abbreviated to a three-letter code (pal for palmitate, myr for myristate, etc.) and incorporated into the synonym for each protein in a similar way to which the letters g (for glycoprotein) and p (for phosphoprotein) are used. For example, the transforming protein of Rous sarcoma virus would be fully represented as myr-pp60$^{v\text{-}src}$. In a similar fashion abbreviations for the other acyl groups reported to modify proteins (Marinetti and Cattieu, 1982; Schmidt, 1984) could be devised.

Materials and methods

[9, 10(n)-^{3}H]Palmitic acid (40 – 60 Ci/mmol) and L-[^{35}S]methionine (> 1000 Ci/mmol) were obtained from Amersham International plc. [9, 10(n)-^{3}H]Myristic acid (10 – 60 Ci/mmol) was from New England Nuclear. Cells used and sources were as follows: CEF from 11-day-old embryos, Mr. J. Wills, Virology Division, NIMR; baby hamster kidney (BHK21-C13), Dr. R.C. Hughes, Biochemistry Division, NIMR; Madin-Darby canine kidney (MDCK) and Madin-Darby bovine kidney (MDBK), Gibco Europe; A431 human colon carcinoma, Dr. P. Goodfellow, ICRF; Rat-1, Dr. K. Quade, NIMR; SV40-transformed Rat-1, Dr. D. Kalderon, NIMR; polyoma virus transformed-Rat-1, Dr. B. Ely, NIMR. CEF (Dr. J. Wyke, ICRF, London) were transformed with the Schmidt-Ruppin strain of Rous sarcoma virus. Cells were routinely grown in Ham's F15 medium containing 10% foetal calf serum (Sera Lab), and supplemented with penicillin/streptomycin in a 95% air-5% CO2 atmosphere. All reagents were Analar or the purest grade available.

Labelling of cells and analysis of acyl proteins

Labelling of subconfluent or newly confluent cultures in 35 mm dishes was performed in normal culture medium supplemented with 5 mM sodium pyruvate using 50 – 100 μCi/ml of the appropriate fatty acid or methionine-depleted medium with 10 – 50 μCi/ml of [^{35}S]methionine. After the labelling period medium was removed, cells were washed twice with ice-cold phosphate buffered saline and lysed in gel loading buffer (2%SDS – 68 mM Tris-HCl pH 6.8 – 10 mM DTT containing 10% glycerol and 0.1% bromophenol blue). After breakage of DNA by repeated pipetting and boiling for 2 – 5 min, samples were analysed on 1 mm thick 5 – 15% SDS-polyacrylamide gradient gels using the buffer system of Laemmli (1970). Fluorography was according to Bonner and Laskey (1974) using preflashed XOmat G or XAR-5 film (Kodak) at −70°C. Protein was assayed by the method of Lowry *et al.* (1951) with the inclusion of 0.5% SDS in each sample. Radioactivity was determined by scintillation counting in a Beckman LS 2800 counter using 299 scintillation cocktail (United Technology Packard).

To test for ester-linked fatty acid gels were treated for 60 min at room temperature with 1 M hydroxylamine-HCl pH 8.0 before fluorography (Magee *et al.*, 1984).

Subcellular fractionation and immunoprecipitation

Lysis and fractionation of Rat-1 cells in discontinuous sucrose gradients was performed as described by Courtneidge *et al.* (1983). Fractions obtained were dissolved in gel loading buffer without bromophenol blue or DTT and assayed for protein. Gel analyses were performed with equal proportions of the fractions to demon-

strate the relative distribution of acyl proteins, and also with equal amounts of protein to allow a qualitative estimate of specific activity of acyl proteins in each fraction.

Immunoprecipitations followed the method of Courtneidge and Smith (1983). Antisera used were: monoclonal mouse-anti-human transferrin receptor (kind gift of Dr. C. Schneider, ICRF, Mill Hill, London); monoclonal mouse-anti-human epidermal growth factor receptor (R1, gift of Dr. M. Waterfield, ICRF, Lincoln's Inn Field, London); rabbit anti-pp36 (Courtneidge *et al.*, 1983), rabbit antibodies reactive against $pp60^{v\text{-}src}$ and $pp60^{c\text{-}src}$ (TBR) (Levinson *et al.*, 1978).

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