# The Majority of Cellular Fatty Acid Acylated Proteins Are Localized to the Cytoplasmic Surface of the Plasma Membrane†

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ABSTRACT: The $BC_3H1$ muscle cell line was previously reported to contain a broad array of fatty acid acylated proteins [Olson, E. N., Towler, D. A., & Glaser, L. (1985) *J. Biol. Chem. 260*, 3784–3790]. Palmitate was shown to be attached to membrane proteins posttranslationally through thiol ester linkages, whereas myristate was attached cotranslationally, or within seconds thereafter, to soluble and membrane-bound proteins through amide linkages [Olson, E. N., & Spizz, G. (1986) *J. Biol. Chem. 261*, 2458–2466]. The temporal and subcellular differences between palmitate and myristate acylation suggested that these two classes of acyl proteins might follow different intracellular pathways to distinct subcellular membrane systems or organelles. In this study, we examined the subcellular localization of the major fatty acylated proteins in $BC_3H1$ cells. Palmitate-containing proteins were localized to the plasma membrane, but only a subset of myristate-containing proteins was localized to this membrane fraction. The majority of acyl proteins were nonglycosylated and resistant to digestion with extracellular proteases, suggesting that they were not exposed to the external surface of the plasma membrane. Many proteins were, however, digested during incubation of isolated membranes with proteases, which indicates that these proteins face the cytoplasm. Two-dimensional gel electrophoresis of proteins labeled with [$^3$H]palmitate and [$^3$H]myristate revealed that individual proteins were modified by only one of the two fatty acids and did not undergo both N-linked myristylation and ester-linked palmitylation. Together, these results suggest that the majority of cellular acyl proteins are routed to the cytoplasmic surface of the plasma membrane, and they raise the possibility that fatty acid acylation may play a role in intracellular sorting of nontransmembranous, nonglycosylated membrane proteins.

A wide range of membrane proteins is modified by the covalent attachment of long-chain fatty acids [for reviews, see Magee and Schlesinger (1982), Schmidt (1983), and Olson (1986)]. Recent studies indicate that at least two distinct pathways exist for protein fatty acid acylation (Magee & Schlesinger, 1982; Schmidt, 1983; Olson, 1986). The best characterized pathway involves esterification of the 16-carbon fatty acid palmitate to a subset of integral membrane proteins. A relatively small number of proteins has also been shown to be acylated with the 14-carbon fatty acid myristate through an amide bond to an amino-terminal glycine (Aitken et al., 1982; Carr et al., 1982; Henderson et al., 1983; Marichildon et al., 1984; Ozols et al., 1984; Buss et al., 1985; Schultz et al., 1985; McIlhinney et al., 1985; Magee et al., 1985; Olson et al., 1985; Olson & Spizz, 1986; Towler & Glaser, 1986). In contrast to palmitylation, myristylation appears to take place during or immediately after translation of acyl proteins and is common to both membrane and cytosolic proteins (Olson et al., 1985; Magee & Courtneidge, 1985; McIlhinney et al., 1985; Olson & Spizz, 1986). Despite the preliminary characterization of these two pathways for fatty acylation, the precise sequence of events involved in processing and intracellular transport of acyl proteins has not been determined nor have the functions of fatty acylation been clearly defined.

The most thoroughly studied acyl proteins have been the envelope virus glycoproteins (Dunphy et al., 1981; Quinn et al., 1983; Schmidt & Schlesinger, 1980, 1979; Schmidt et al., 1979) and the retrovirus transforming proteins (Carr et al., 1982; Schultz et al., 1985; Sefton et al., 1982) because of their high levels of expression in virus-infected cells. The characteristics of normal cellular acyl proteins, which represent a very minor fraction of membrane proteins, have not been thoroughly examined. To investigate the mechanisms involved in biogenesis of cellular acyl proteins, we used the $BC_3H1$ muscle cell line, which contains a broad array of proteins acylated with palmitate and myristate (Olson et al., 1984, 1985; Olson & Spizz, 1986). Acylation in these cells is highly specific with respect to fatty acyl donor and protein acceptor. Analysis of the characteristics of the two major classes of acyl proteins in $BC_3H1$ cells should, therefore, provide a more general understanding of fatty acylation than might be obtained from examination of an individual acyl protein.

To begin to define the intracellular pathways followed by acyl proteins, we examined the subcellular distribution of the major palmitate- and myristate-containing proteins in $BC_3H1$ cells. The majority of these were nonglycosylated but localized to the plasma membrane. In addition, these proteins did not appear to be exposed to the extracellular milieu, since they were resistant to digestion with external proteases. Several acyl proteins were, however, digested during incubation of isolated membranes with proteases, indicating that these proteins are exposed to the cytoplasmic face of the plasma membrane. These results demonstrate that the majority of cellular fatty acylated proteins are targeted to the plasma membrane but are not transmembrane glycoproteins and raise the interesting possibility that fatty acylation may serve as a mechanism for sorting of some nonglycosylated proteins to the plasma membrane.

## Materials and Methods

*Cell Culture and Labeling Conditions.* The $BC_3H1$ mouse muscle cell line (Schubert et al., 1974) was grown on 10-cm

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Falcon tissue culture dishes as described previously (Olson et al., 1983a). For lipid-labeling experiments, confluent cultures containing approximately $1 \times 10^7$ cells were incubated in DME[1] containing 10% delipidated and dialyzed fetal calf serum, L-glutamine (0.1 μg/mL), 5 mM pyruvate, and [9,10-$^3$H]palmitic acid (20–40 Ci/mmol, New England Nuclear) or [9,10-$^3$H]myristic acid (55 Ci/mmol, New England Nuclear) for the labeling periods specified in each experiment. Glycoproteins were labeled with 100 μCi/mL of D-[2-$^3$H]-mannose (30–60 Ci/mmol, ICN) in media containing DME with 0.2 mM α-D-glucose. For labeling RNA, cells were incubated with 50 μCi of [5-$^3$H]uridine (30 Ci/mmol, Amersham) in DME with 10% dialyzed fetal calf serum.

To harvest cells after labeling, the medium was removed and the cultures were rinsed twice at 4 °C in NaCl/$P_i$ containing 15 mM iodoacetamide as a protease inhibitor. Cells were scraped into the same buffer and collected by centrifugation as described (Olson et al., 1983a).

*Subcellular Fractionation.* To determine the subcellular distribution of palmitate- and myristate-containing proteins, a modification of the Percoll gradient procedure described by Hyman and Froehner (1983) was used. In brief, the cell pellet, harvested as described above, was resuspended in 2 mL of TEA/sucrose (1 mM triethanolamine, 0.25 M sucrose, pH 7.2) and homogenized for 10 s in a Tekmar Tissuemizer (Tekmar, Cincinnati, OH). Three additional milliliters of TEA/sucrose was mixed with the homogenate, and a nuclear pellet was prepared by centrifugation for 10 min at 1000*g*. The supernatant was removed and the nuclear pellet resuspended in 5 mL of TEA/sucrose and centrifuged for 10 min at 1000*g*. After the two postnuclear supernatants were combined, membranes were collected by centrifugation for 60 min at 33000*g*. The membrane pellet was resuspended in 1.0 mL of 15% isoosmotic Percoll (v/v in isoosmotic Percoll and TEA/sucrose) and layered over a 2.5-mL cushion of 2.5 M sucrose in a 60 Ti centrifuge tube. An additional 20 mL of 15% Percoll was placed above the sample. The gradient was centrifuged for 20 min at 45000*g* with low-speed acceleration and no brake. One-milliliter fractions were collected from the top of the gradient. A density marker bead kit (Pharmacia Fine Chemicals, Piscataway, NJ) was used to determine densities of the gradient fractions. Following determination of membrane marker activities in the gradient, fractions were pooled and diluted with NaCl/$P_i$. The Percoll was removed from the samples by centrifugation for 3 h at 100000*g*. Membranes lying above the Percoll pellet were resuspended in sample buffer and analyzed by NaDod$SO_4$–polyacrylamide gel electrophoresis.

For subcellular fractionation of membranes on continuous sucrose gradients, cells were isolated as described above. The cell pellet was resuspended in 5 mL of TEA/sucrose, placed on ice for 10 min, and homogenized with 15 strokes in a tight-fitting Dounce homogenizer. The homogenate was centrifuged for 10 min at 1000*g*, and the nuclear pellet was resuspended in 5 mL of TEA/sucrose and centrifuged again. The postnuclear supernatants, obtained from the two low-speed spins, were pooled and centrifuged for 1 h at 33000*g*. The membrane pellet was resuspended in 1.0 mL of 55% sucrose (w/v in 1 mM EDTA and 1 mM Tris, pH 8.0). The sample was layered over a 0.5-mL cushion of saturated sucrose in an SW41 centrifuge tube. An 11-mL continuous-density gradient of 25–45% (w/v) sucrose was placed over the sample, and the gradient was centrifuged for 16.5 h at 75000*g*; 0.5-mL fractions were collected from the top of each gradient. After determination of membrane marker activities, membranes from pooled fractions were diluted with NaCl/$P_i$ and collected by centrifugation at 100000*g* for 1 h. The membrane pellet was resuspended in sample buffer and analyzed by NaDod$SO_4$–polyacrylamide gel electrophoresis.

*Enzyme Assays.* Cytochrome oxidase activity was assayed according to the method of Cooperstein and Lazarow (1951). NAβGase activity was measured as described by Kaplan and Jamieson (1977). Membranes were mixed with a 2× reaction mixture containing 5 mM *p*-nitrophenyl *N*-acetyl-β-glucosaminide, 0.2 M citric acid, and 0.4 M $Na_2HPO_4$, pH 4.5. After 30-min incubation at 37 °C, the reaction was terminated by the addition of 0.75 mL of 0.25 M glycine, pH 10.2. After centrifugation for 5 min in a table-top Eppendorf centrifuge, the amount of sialic acid hydrolyzed was determined from the absorbance at 400 nm with $E_{400nm} = 1.77 \times 10^{-4}$ $M^{-1}$ $cm^{-1}$. Total activity was determined by the same procedure with 0.2% Triton X-100 in the 2× reaction mixture. Latent activity represents the difference between activity in the presence and absence of Triton X-100. All enzyme assays were linear with respect to time and membrane protein concentration.

*Cell Surface Iodination.* Proteins exposed to the cell surface were iodinated by lactoperoxidase–glucose oxidase catalyzed iodination (Morrison, 1974).

*Protease Digestions.* For digestion of extracellular proteins, cells were labeled with either [$^3$H]palmitate or [$^3$H]myristate for 8 h. At the end of the labeling period, cells were treated for 15 min at 37 °C in the presence or absence of trypsin or Pronase, each at 250 μg/mL as specified. Digestions were terminated by addition of soybean trypsin inhibitor (1 mg/mL), and cells were harvested in DME containing 20% fetal calf serum to quench proteolytic activity. Cells were then collected by centrifugation and washed once with NaCl/$P_i$. Extracts were prepared as described previously (Olson et al., 1983a), and labeled proteins were analyzed by NaDod$SO_4$–polyacrylamide gel electrophoresis.

For protease digestion of isolated membranes, cells were labeled with [$^3$H]palmitate or [$^3$H]myristate for 8 h. A crude membrane pellet was prepared and treated for 30 min at 37 °C in the presence or absence of trypsin or Pronase, each at 250 μg/mL, as specified. Samples were then analyzed by NaDod$SO_4$–polyacrylamide gel electrophoresis.

*Protein Determination.* Membrane protein was determined by the method of Bramhall et al. (1969) for Percoll gradient fractions and by the method of Bradford et al. (1976) for sucrose gradient fractions.

*ACh Receptor Assay.* The ACh receptor was measured by the specific binding of $^{125}$I-α-bungarotoxin as described (Olson et al., 1983b). To ensure that only the surface receptor pool was labeled, cultures were incubated with $^{125}$I-α-bungarotoxin for 20 min. More than 90% of total binding was specific and could be blocked by preincubation of cultures with 0.1 μM unlabeled α-bungarotoxin.

*Electrophoresis.* Electrophoresis of 10% NaDod$SO_4$–polyacrylamide gels was performed as described (Olson et al., 1983a). For two-dimensional gel electrophoresis, samples were resuspended in 75 μL of IEF sample [9.95 M urea, 4.0% Nonidet P-40, 1.6% pH 5–7 ampholytes (Bio-Rad Laboratories, Richmond, CA)], 0.4% pH 3–10 ampholytes (Bio-Rad),

[1] Abbreviations: BTX, α-bungarotoxin; DME, Dulbecco's minimal essential medium; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NAβGase, *N*-acetyl-β-glucosaminidase; IEF, isoelectric focusing; NaCl/$P_i$, phosphate-buffered saline, 0.14 M NaCl and 10 mM sodium phosphate, pH 7.2; NaDod$SO_4$, sodium dodecyl sulfate; TEA, triethanolamine; NADH, reduced nicotinamide adenine dinucleotide.

100 mM dithiothreitol, and 0.3% $NaDodSO_4$ (Garrels, 1979). Electrophoresis of IEF gels was performed as described by O'Farrell (1975). The second dimensions were run on 10% $NaDodSO_4$–polyacrylamide gels. After staining and destaining, gels were treated with Enhance (New England Nuclear) according to the manufacturer's instructions.

## Results

*Subcellular Fractionation of $BC_3Hl$ Cells.* To determine the subcellular localization of cellular acyl proteins, we developed a fractionation procedure that would separate major subcellular organelles. First, a postnuclear membrane fraction was prepared from $BC_3Hl$ cells. This was further subfractionated on Percoll rate zonal gradients, and gradient fractions were assayed for membrane markers specific for the plasma membrane, mitochondria, lysosomes, and endoplasmic reticulum (Figure 1).

To identify plasma membranes, the acetylcholine receptor was measured by incubating cultures with $^{125}I$-$\alpha$-bungarotoxin for 20 min before harvesting. An incubation of this length is too short to allow receptor internalization and allows labeling of only the cell surface acetylcholine receptor pool (Hyman & Frochner, 1983). Plasma membranes were distributed as a single peak near the top of the gradient that coincided with the peak of membrane protein (Figure 1A). Cytochrome oxidase activity, a mitochondrial marker, was distributed primarily in fractions 21–25, near the bottom of the gradient. A minor peak of cytochrome oxidase activity was observed also in the light-density region of the gradient in the same fractions as the major peak of membrane protein. The activity profile of NA$\beta$Gase, a marker for lysosomes, also showed peaks in the gradient's high- and low-density regions (Figure 1B). NA$\beta$Gase activity of the dense material was ~60% latent, whereas activity in the lower density fractions was almost all nonlatent. The high percentage of nonlatent enzyme activity suggested that the membrane subfractionation procedure resulted in breakage of lysosomes. Attempts to maintain latency of NA$\beta$Gase activity and to localize the distribution of cytochrome oxidase and NA$\beta$Gase activities to a single region of the gradient by altering salt concentration of the buffers or varying the procedures for homogenization were unsuccessful. Membranes from the rough endoplasmic reticulum, measured by [$^3$H]uridine labeling, were distributed as a major peak in the light region of the gradient and were indistinguishable from the peak of membrane protein and $^{125}I$-$\alpha$-bungarotoxin binding (Figure 1C). Thus, subfractionation of $BC_3Hl$ membranes on Percoll rate zonal gradients resulted in clear separation of plasma membranes from mitochondria and a partial separation from lysosomes, but the technique did not separate plasma membranes from rough endoplasmic reticulum.

Since plasma membranes and rough endoplasmic reticulum were not separable on Percoll gradients, we used a continuous-density sucrose gradient to fractionate these two membrane systems. The membrane fraction prepared by Dounce homogenization was layered over a 65% (w/v) surose cushion and overlayed with a 25–45% (w/v) continuous-density sucrose gradient. Gradients were centrifuged to equilibrium, and membrane markers were assayed across the gradient (Figure 2). In contrast to the distribution of membrane protein on Percoll gradients, membrane protein on sucrose gradients was distributed across the entire gradient with a peak in the high-density region (Figure 2C). $^{125}I$-$\alpha$-Bungarotoxin exhibited a broad distribution across the low-density region of the gradient with a minor peak in the high-density fractions that coincided with the peak of membrane material (Figure 2A). Cytochrome oxidase activity was distributed almost exclusively

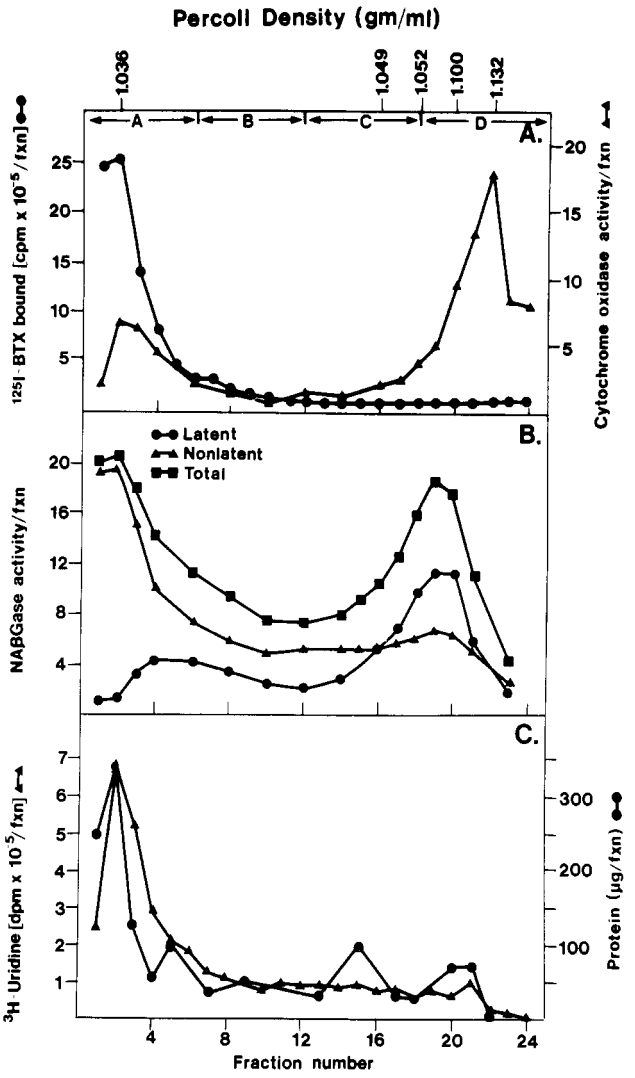


FIGURE 1: Fractionation of membranes from $BC_3Hl$ cells on Percoll gradients. Membranes from a postnuclear supernatant were isolated and fractionated on Percoll gradients, and gradient fractions were assayed for membrane marker activities as described under Materials and Methods. (A) $^{125}I$-$\alpha$-Bungarotoxin binding and cytochrome oxidase activity; (B) total, latent, and nonlatent NA$\beta$Gase; (C) membrane protein and [$^3$H]uridine incorporation. Cytochrome oxidase activity is expressed as relative change in $A_{550}$/min. NA$\beta$Gase activity is expressed as nmol of *p*-nitrophenyl *N*-acetyl-$\beta$-glucosaminide hydrolyzed/min. Similar distributions of membrane markers were obtained in five separate experiments. A–D at the top of the figure represent the gradient fractions that were pooled for analysis of fatty acid labeled protein by $NaDodSO_4$–polyacrylamide gel electrophoresis (see Figure 3).

in the high-density region of the gradient (Figure 2A) as was NA$\beta$Gase activity (Figure 2B). In contrast to the distribution of rough endoplasmic reticulum membranes on Percoll gradients, these membranes were localized to the high-density region of continuous-density sucrose gradients, fractions 21–24 (Figure 2C). Thus, subfractionation of $BC_3Hl$ membranes on continuous-density sucrose gradients resulted in clear separation of plasma membranes from lysosomes, mitochondria, and endoplasmic reticulum but did not resolve the latter three organelles from each other. The combination of the two types of gradients enabled us to localize the major acyl proteins in $BC_3Hl$ cells to specific membrane systems (see below).

*Subcellular Localization of Acylated Proteins.* To determine the subcellular localization of fatty acylated proteins, $BC_3Hl$ cells were labeled for 8 h with [$^3$H]myristate or [$^3$H]palmitate. At the end of the labeling period, membranes

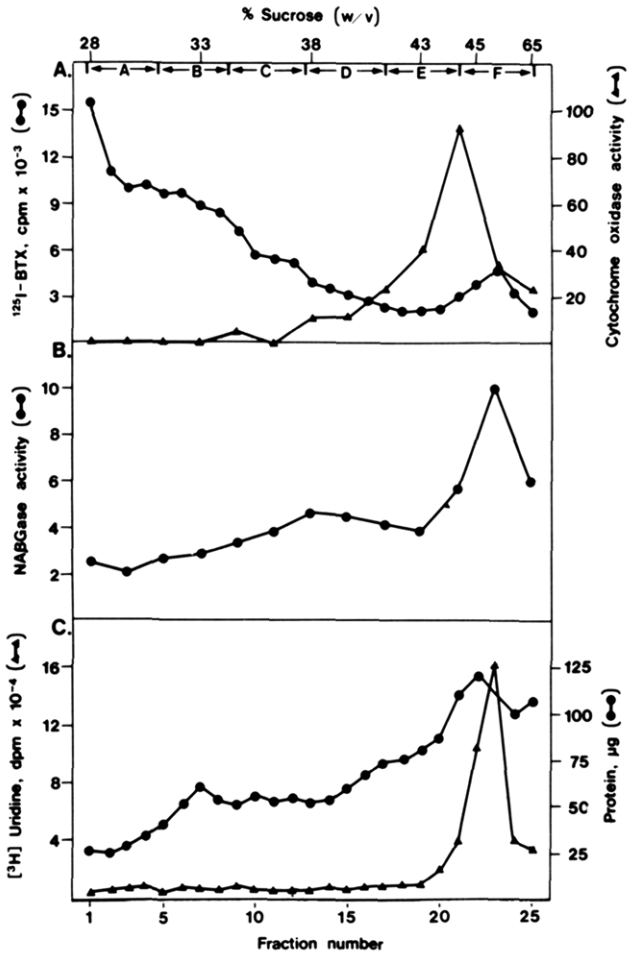


FIGURE 2: Fractionation of membranes from $BC_3HI$ cells on continuous-density sucrose gradients. Membranes from a postnuclear supernatant were isolated and fractionated on continuous-density (20–45%, w/v) sucrose gradients, and gradient fractions were assayed for membrane marker activities as described under Materials and Methods. (A) $^{125}$I-α-Bungarotoxin binding and cytochrome oxidase activity; (B) NAβGase activity; (C) membrane protein and [$^3$H]uridine incorporation. Cytochrome oxidase activity is expressed as percentage of total activity in the gradient. NAβGase activity is expressed as nmol of *p*-nitrophenyl *N*-acetyl-β-glucosaminide hydrolyzed/min. Similar distributions of membrane markers were obtained in five separate experiments. A–F at the top of the figure represent the gradient fractions that were pooled for analysis of fatty acid labeled protein by $NaDodSO_4$–polyacrylamide gel electrophoresis (see Figure 4).

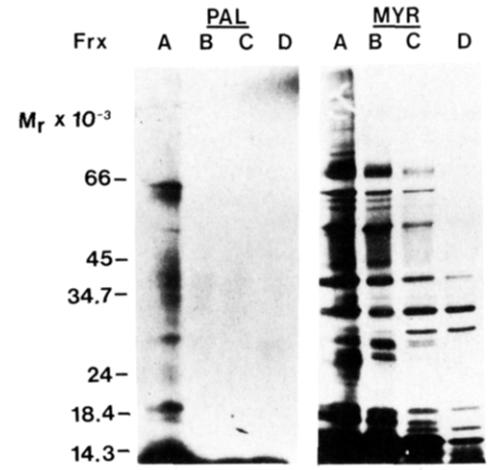


FIGURE 3: $NaDodSO_4$–polyacrylamide gel electrophoresis of fatty acylated proteins from Percoll gradient fractions. $BC_3HI$ cells were labeled separately with [$^3$H]palmitate or [$^3$H]myristate, and membranes were isolated and fractionated on Percoll gradients as described under Materials and Methods. Gradient fractions were pooled into four fractions shown in Figure 1A. Membranes contained in fractions A–D were collected by centrifugation, and equivalent quantities of protein from each fraction were analyzed by electrophoresis on 10% $NaDodSO_4$–polyacrylamide gels. Note that all major palmitylated proteins and the majority of myristylated proteins are localized to fraction A, which is enriched for plasma membranes.

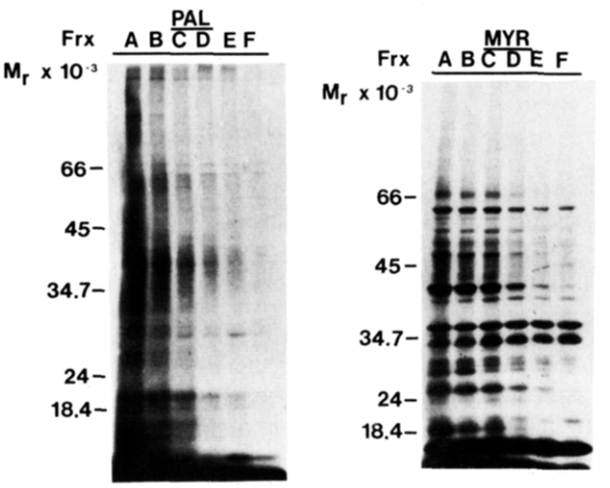


FIGURE 4: $NaDodSO_4$–polyacrylamide gel electrophoresis of fatty acylated proteins from continuous-density sucrose gradients. $BC_3HI$ cells were labeled separately with [$^3$H]palmitate or [$^3$H]myristate, and membranes were isolated and fractionated on continuous-density sucrose gradients as described under Materials and Methods. Gradient fractions were pooled into six fractions shown in Figure 2A. Membranes contained in fractions A–E were collected by centrifugation, and equivalent quantities of proteins from each fraction were analyzed by electrophoresis on 10% $NaDodSO_4$–polyacrylamide gels. Note that all major palmitylated proteins and the majority of myristylated proteins are localized to fractions A–C, which are enriched for plasma membranes.

were prepared and fractionated on Percoll gradients, as described above. Gradient fractions were pooled into four subfractions containing the following regions of each gradient: A, fractions 1–7; B, fractions 8–14; C, fractions 15–21; and D, fractions 22–25. Membranes were concentrated by centrifugation, and the distribution of fatty acylated proteins across the gradient was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 3). Palmitate-containing proteins were localized almost exclusively to the low-density region of the gradient (subfraction A). The distribution of these proteins paralleled the distribution of $^{125}$I-α-bungarotoxin and [$^3$H]uridine (see Figure 1). A number of myristate-labeled proteins were also localized primarily to the low-density region of the gradient; however, a subset of myristylated proteins was found in other regions of the gradient. In particular, two major myristylated proteins with $M_r$ ~30K and 35K were distributed across the entire gradient.

The distributions of the palmitylated proteins and the majority of the myristylated proteins across the Percoll gradient suggested that they were localized to the plasma membrane, the rough endoplasmic reticulum, or both. To distinguish between these possibilities, cells were labeled with [$^3$H]palmitate or [$^3$H]myristate, membranes were prepared and fractionated on continuous-density sucrose gradients, and the distribution of acylated proteins across the gradients was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 4). The majority of [$^3$H]palmitate-labeled proteins was localized to the light-density region of the gradient. The distribution of these proteins coincided with the distribution of $^{125}$I-α-bungarotoxin (see Figure 2) indicating that they were targeted to the plasma membrane. Many [$^3$H]myristate-labeled proteins also appeared to be localized to the plasma

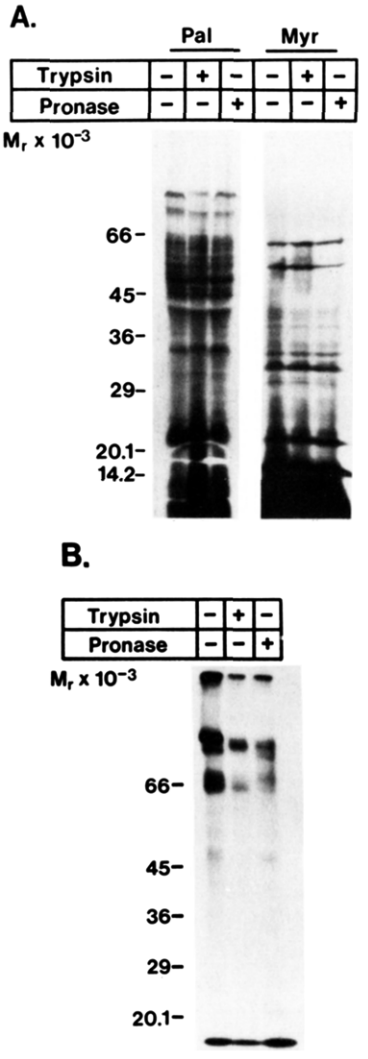


FIGURE 5: Sensitivity of fatty acylated proteins to extracellular protease digestion. $BC_3H1$ cells were metabolically labeled with [$^3$H]palmitate or [$^3$H]myristate (A) or were labeled by iodination of cell surface proteins (B) as described under Materials and Methods. At the end of the labeling period, cultures were treated or not treated for 15 min at 37 °C with trypsin (250 μg/mL) or Pronase (250 μg/mL) as specified, and extracts were prepared and analyzed by electrophoresis on 10% $NaDodSO_4$–polyacrylamide gels. Note that the major acylated proteins are resistant to digestion, whereas several cell surface $^{125}$I-labeled proteins are susceptible to digestion with extracellular proteases.

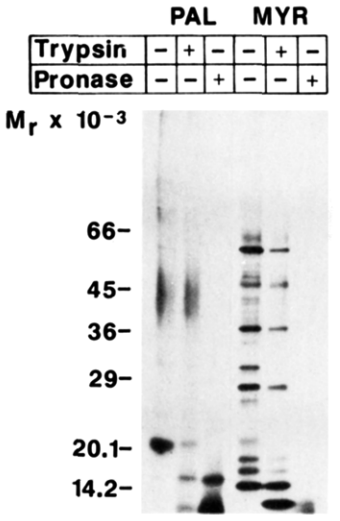


FIGURE 6: Sensitivity of fatty acylated proteins in isolated membranes to protease digestion. $BC_3H1$ cells were labeled with [$^3$H]palmitate or [$^3$H]myristate, as described under Materials and Methods. At the end of the labeling period, total cell membranes were isolated and digested for 30 min at 37 °C with or without trypsin (250 μg/mL) or Pronase (250 μg/mL), as specified. Labeled proteins were then analyzed by electrophoresis on 10% $NaDodSO_4$–polyacrylamide gels. Addition of enzymes followed by immediate termination of the digestion had no apparent effect on mobility of acyl proteins (data not shown). The intensity of labeled bands in this experiment is reduced compared to that of those in Figure 5 due to a lower level of incorporation of $^3$H-labeled fatty acids into proteins during the labeling period.

membrane. However, the distribution of myristylated proteins was not as restricted to the low-density region of the sucrose gradients as it was in Percoll gradients. The myristylated proteins with $M_r$'s ~30K and 35K were found at approximately the same levels in all membrane fractions. Thus, palmitate-acylated proteins are directed to the plasma membrane, whereas myristylated proteins exhibit a broader subcellular distribution.

*The Majority of Membrane-Associated Acyl Proteins Are Not Susceptible to Extracellular Proteases.* To determine whether the major palmitate- and myristate-containing proteins, which were localized to the plasma membrane, were exposed to the cell exterior, $BC_3H1$ cells were labeled for 7 h with [$^3$H]palmitate or [$^3$H]myristate, after which time they were treated or not treated with trypsin or Pronase to digest proteins exposed at the cell surface. Samples were then analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis followed by fluorography. To ensure that this treatment was sufficient for digestion of cell surface polypeptides, a separate set of cultures was labeled by lactoperoxidase–glucose oxidase catalyzed iodination. This technique specifically labels polypeptides exposed at the cell surface. As shown in Figure 5A, the major acyl proteins were resistant to digestion with extracellular proteases, suggesting that they are not exposed to the cell surface. Two palmitate-acylated proteins at 65 and 80 kDa were altered slightly by trypsin digestion, which may indicate that they were partially exposed at the cell surface. In contrast, several abundant $^{125}$I-labeled cell surface polypeptides were susceptible to proteolytic digestion (Figure 5B). These results suggest that the majority of acyl proteins in the plasma membrane is not exposed to the extracellular milieu or is protease resistant. Because these proteins are nonglycosylated, their protease resistance cannot be attributed to protection by attached carbohydrate.

To determine whether acylated proteins were exposed to the inner surface of the plasma membrane, $BC_3H1$ cells were labeled with [$^3$H]myristate and [$^3$H]palmitate and a postnuclear membrane fraction was isolated. Membranes were digested or not digested with trypsin or Pronase, and the patterns of labeled proteins were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. As shown in Figure 6, several acyl proteins were susceptible to proteolytic digestion in isolated membranes. This suggests that these acyl proteins in the plasma membrane face the cytoplasmic surface of the membrane.

*Two-Dimensional Gel Electrophoresis of Acylated Proteins.* As shown in Figures 3–6, a number of palmitate- and myristate-labeled proteins exhibit similar molecular weight after electrophoresis on one-dimensional $NaDodSO_4$–polyacrylamide gels. Comigration of palmitate- and myristate-labeled proteins could be due to single acyl proteins with both palmitate and myristate acylation sites or to multiple acyl proteins with similar molecular weights. Because we would ultimately like to know whether palmitate- and myristate-acylated proteins follow the same or different intracellular pathways, we attempted to determine whether some polypeptides undergo both

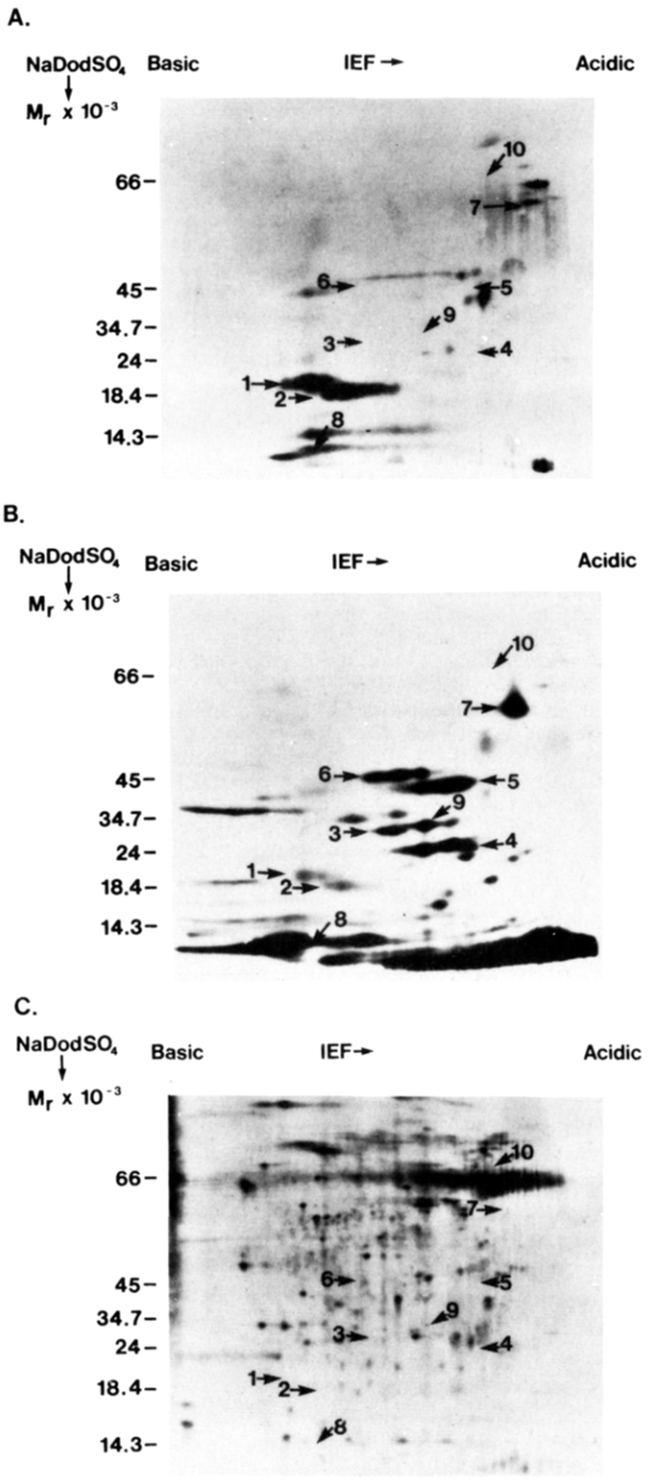


FIGURE 7: IEF/NaDodSO$_4$–polyacrylamide gel electrophoresis of metabolically labeled proteins. $BC_3H1$ cells were labeled separately for 8 h with [$^3$H]mannose, [$^3$H]palmitate, or [$^3$H]myristate. At the end of the labeling period, membranes were isolated from the post-nuclear supernatant fraction and analyzed by IEF/NaDodSO$_4$–polyacrylamide gel electrophoresis as described under Materials and Methods. (A) [$^3$H]Palmitate-labeled proteins; (B) [$^3$H]myristate-labeled proteins; (C) [$^3$H]mannose-labeled proteins. The major palmitate- and myristate-labeled proteins are indicated with arrows. Note that none of the major acyl proteins correspond to [$^3$H]-mannose-labeled glycoproteins.

N-linked myristylation and ester-linked palmitylation by using two-dimensional gel electrophoresis. As shown in Figure 7, the patterns of proteins labeled with [$^3$H]palmitate and [$^3$H]myristate were distinct. Many acylated proteins appeared to migrate as multiple species in the IEF dimension, suggesting the existence of different forms of these proteins (see proteins 1–6 and 8). The major palmitate-labeled proteins, which exhibit $M_r$ ~20K and 22K, seemed to be labeled to a low level with [$^3$H]myristate (parts A and B of Figure 7 compare proteins 1 and 2). Since about 10–20% of myristate is converted to palmitate during an 8-h labeling period (Olson et al., 1985), this label is probably in the form of palmitate. These results demonstrate that in $BC_3H1$ cells individual acyl proteins do not undergo both myristylation and palmitylation.

Previously, we reported (Olson & Spizz, 1986) that more than 99% of the palmitate- and myristate-containing proteins in $BC_3H1$ cells were resistant to digestion with *N*-acetyl-$\beta$-glucosaminidases H and F and did not bind to concanavalin A, indicating that these proteins are not N-glycosylated. These results contrasted with those of Magee and Courtneidge (1985), who reported that in chicken embryo fibroblasts many palmitylated proteins were glycoproteins. To address this issue, we compared [$^3$H]mannose-labeled proteins with [$^3$H]palmitate- and [$^3$H]myristate-labeled proteins by two-dimensional gel electrophoresis. None of the major acyl proteins in $BC_3H1$ cells comigrated with mannose-labeled glycoproteins (Figure 7, compare C with A and B). Since the abundance of these acyl proteins relative to the glycoproteins is difficult to determine, the failure to detect single polypeptides labeled both with $^3$H-labeled fatty acids and [$^3$H]mannose could be due to differences in abundance. We believe this possibility is unlikely, however, because several acyl proteins could be visualized by Coomassie blue staining of two-dimensional gels (data not shown) and would, therefore, be detected in [$^3$H]-mannose-labeled extracts if they were glycosylated. Combined with previous studies (Olson & Spizz, 1986), these results support the conclusion that the majority of acyl proteins in $BC_3H1$ cells are not N-linked glycoproteins.

## Discussion

The results of this study extend our understanding of the biogenesis of acylproteins in three important respects. First, they show that the major cellular palmitate-containing proteins are localized to the plasma membrane. A subset of myristylated proteins is also localized to the plasma membrane, but the distribution of this class of acyl proteins is not as restricted as that of the palmitate-containing proteins. Second, most cellular acyl proteins are nonglycosylated and not exposed to the exterior of the cell. Finally, the results presented in Figure 7 show that individual proteins are modified by attachment of either palmitate or myristate, but not both.

The localization of nonglycosylated acyl proteins to the plasma membrane raises interesting questions about the intracellular pathways followed by this unique class of proteins and about the subcellular localization of the protein fatty acyl transferases. Several cell surface glycoproteins have been shown to be acylated with palmitate in the cis-Golgi apparatus or transitional elements of the endoplasmic reticulum 5–15 min after synthesis (Dunphy et al., 1981; Quinn et al., 1983; Schmidt & Schlesinger, 1980). The results reported here and in previous studies (Olson & Spizz, 1986) demonstrate, however, that only a small fraction of cellular palmitate-containing proteins are glycosylated. The majority of palmitate-containing proteins do not contain carbohydrate and can acquire covalently bound fatty acid up to 180 min after translation (Olson & Spizz, 1986). These results suggest that multiple pathways and perhaps multiple enzymes may exist for palmitate acylation of glycosylated and nonglycosylated membrane proteins.

Recently, ankyrin and p21*ras* were shown to be palmitylated (Staufenbiel & Lazarides, 1986; Sefton et al., 1982). Both

of these nonglycosylated membrane proteins seem to be synthesized on free polysomes and subsequently transported to the cytoplasmic surface of the plasma membrane. Palmitate attachment to p21*ras* occurs very close to the time of membrane association (Sefton et al., 1982). The similarities between ankyrin and p21*ras* and the palmitylated proteins described in this study suggest that these proteins may be directed to the plasma membrane and fatty acylated by the same mechanism. Whether these proteins are acylated immediately before or after membrane binding is unknown. The highly specific localization of palmitylated proteins to the plasma membrane suggests, however, that the palmityl acyltransferases may also be localized to this membrane fraction.

In contrast to palmitylation, myristylation is an early modification that takes place cotranslationally or within seconds thereafter (Olson & Spizz, 1986). The only myristylated protein to be examined in detail is $p60^{src}$, which is synthesized on free polysomes, myristylated at its amino terminus (Buss & Sefton, 1985; Schultz et al., 1985), and transported to the plasma membrane within 15 min following translation (Levinson et al., 1980). These kinetics for myristylation suggest that the myristyl acyltransferase may be localized to the cytosol rather than to a specific membrane fraction. Myristylation of proteins before membrane association is consistent with the distribution of these proteins in a number of membrane fractions because this acylation mechanism would not require transport through a specific membrane system or organelle. Murine leukemia membrane-associated protein p15 (Henderson et al., 1983), the catalytic subunit of cAMP-dependent protein kinase (Carr et al., 1982), NADH cytochrome $b_5$ reductase (Ozols et al., 1984), and calcineurin b (Aitken et al., 1982) have also been shown to be myristylated. All of these proteins are nonglycosylated and nontransmembranous.

The plasma membrane represents a minor fraction of cellular membranes. Therefore, targeting of proteins from the cytoplasm to this membrane fraction requires a highly specific sorting mechanism. Recent studies on p21*ras* and $p60^{src}$ suggest that fatty acylation may provide a mechanism for routing nonglycosylated proteins to the cytoplasmic face of the plasma membrane. p21*ras* proteins are palmitylated on a cysteine residue near their C-termini. Using a series of mutants containing deletions at or near the C-terminus of p21*ras*, Willumsen et al. (1984) demonstrated that nonacylated p21*ras* proteins did not associate with membranes and did not cause transformation. Similarly, studies by Hanafusa and co-workers using $p60^{src}$ mutants have shown that deletion or modification of the amino-terminal domain of $p60^{src}$ results in an absence of myristylation and that nonacylated $p60^{src}$ does not associate with membranes and does not cause transformation (Cross et al., 1984; Pellman et al., 1985). These investigators also constructed fusion genes in which the nucleotide sequence encoding the first 14 amino acids of $p60^{src}$ was fused to the *fps* gene of the F36 derivative of Fujinami sarcoma virus or to the chimpanzee $\alpha$-globin gene (Pellman et al., 1985a). After transfection of chicken embryo fibroblasts with the chimeric genes, the fusion proteins were myristylated and localized to the plasma membrane.

The precise mechanism whereby a covalently bound lipid directs a protein to a specific membrane is unclear. The fatty acid moiety appears to be more than a hydrophobic anchor that is simply partitioned into the membrane bilayer. If this were the case, one would not expect the highly specific localization of many acylated proteins to the plasma membrane. More likely, covalently bound fatty acid acts in concert with a specific domain of an acyl protein to be correctly routed to a particular organelle. The results presented here provide a foundation for future studies of the precise sequence of events involved in biogenesis, processing, and intracellular transport of cellular acyl proteins to the plasma membrane and for studies designed to determine the role(s) covalent fatty acid play(s) in routing proteins to this specific subcellular location.

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**Registry No.** $CH_3(CH_2)_{14}CO_2H$, 57-10-3; $CH_3(CH_2)_{12}CO_2H$, 544-63-8.

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# Scanning Calorimetric Study of Fully Hydrated Asymmetric Phosphatidylcholines with One Acyl Chain Twice as Long as the Other†

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ABSTRACT: The asymmetric C(18):C(10)PC molecules are known by X-ray diffraction to self-assemble, in excess water, into a lamellar structure known as the mixed interdigitated bilayer at $T < T_m$. In this structure, the long C(18)-acyl chain is interdigitated fully across the entire hydrocarbon width of the bilayer, while the shorter C(10)-acyl chain, which is about half as long as the C(18)-acyl chain, packs end to end with a C(10)-acyl chain of another lipid molecule in the opposing bilayer leaflet. We have synthesized the following asymmetric phosphatidylcholines (PC's): C(16):C(9)PC, C(16):C(10)PC, C(18):C(10)PC, C(18):C(11)PC, C(20):C(11)PC, C(20):C(12)PC, C(22):C(12)PC, C(22):C(13)PC, C(8):C(18)PC, and C(10):C(22)PC. These 10 asymmetric phosphatidylcholines have a common characteristic; i.e., the length of the longer extended acyl chain is about twice as long as that of the shorter acyl chain. On the basis of the known lamellar structure of C(18):C(10)PC, we anticipate that these asymmetric phosphatidylcholines will also form mixed interdigitated bilayers. We have employed high-resolution differential scanning calorimetry (DSC) to investigate the thermotropic behavior of liposomes prepared from these asymmetric phosphatidylcholines. If our anticipation is correct, one would find that the thermodynamic data ($T_m$, $\Delta H$, or $\Delta S$) associated with the main thermal phase transitions of these asymmetric phosphatidylcholine dispersions will fit into a continuous curve as they are plotted as a function of the hydrocarbon width of the putative mixed interdigitated bilayer. Experimental data presented in this paper indeed bear this out. For comparison, a DSC study of multilamellar dispersions prepared from a series of saturated symmetric phosphatidylcholines has also been carried out. The thermodynamic data associated with the main phase transition of these symmetric phosphatidylcholine dispersions are shown to be distinctively different from those of asymmetric phosphatidylcholine species with the same molecular weight. Saturated symmetric phosphatidylcholines are well-known to form normal noninterdigitated bilayers in excess water. When thermodynamic data are plotted against the hydrocarbon width of the normal bilayer, they all fall on a smooth continuous curve. This curve, however, is distinctively different from that fitted by the corresponding data derived from liposomes of asymmetric phosphatidylcholines. Our results can thus be taken as strong evidence to argue for the formation of mixed interdigitated bilayers by the asymmetric phosphatidylcholines under study.

Recently, considerable interest has been placed on phospholipids which, under appropriate conditions, self-assemble into interdigitated bilayers as revealed by the X-ray diffraction technique (Ranck et al., 1977; Hauser et al., 1980; Ranck & Tocanne, 1982; McDaniel et al., 1983; Serrallalch et al., 1983; McIntosh et al., 1984; Hui et al., 1984; Jain et al., 1985; Ruocco et al., 1985; Hui & Huang, 1986; Pascher & Sundell, 1986; Pascher et al., 1986). Among the various interdigitated bilayers, the mixed interdigitated packing model is perhaps most unique, since the acyl chains in the bilayer core are observed to be interdigitated in both the gel and the liquid-crystalline state (McIntosh et al., 1984; Hui et al., 1984).

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