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# 12-*O*-Tetradecanoyl phorbol 13-acetate stimulates the myristylation of an ~82 kDa protein in HL-60 cells

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We have studied protein acylation using [$^3$H]myristate in the two leukemia cell lines HL-60 and HL-60 Blast II. The latter is a variant which does not differentiate after treatment with 12-*O*-tetradecanoyl phorbol 13-acetate (TPA). The acylation profiles of the two cell lines as examined by SDS-PAGE differed. TPA induced the myristylation of an ~82 kDa protein in the sensitive cells, but not in the resistant cells. Myristic acid was shown to be covalently linked to these proteins. Analysis of the cell lipids labelled with [$^3$H]myristate showed that in contrast to observations with the proteins, the changes induced by TPA were observed in both TPA-sensitive and TPA-resistant cells. We conclude that the induction of myristylation may be an important step in the mechanism of differentiation.

Fatty acid; Phorbol ester; Differentiation; (HL-60 promyelocytic leukemia)

## 1. INTRODUCTION

Covalent attachment of fatty acids to proteins has been demonstrated in various cell lines [1–3]. These studies have defined the specificity of the fatty acid acylation with respect to acyl chain length and type of linkage and suggest that the bound fatty acids may act to anchor the protein in the lipid bilayer.

More recently, the retroviral oncogene-encoded protein of several transforming viruses has been shown to be acylated [4–7]. In this case, there is a molecule of myristic acid covalently bound to the amino-terminal of the oncogene product [4,6,7].

Although this acylation step is crucial for both transformation and association of the protein with the membrane, soluble forms are also myristylated [8]. Thus, the role of acylation in carcinogenesis remains to be defined.

We have been studying differentiation in the human promyelocytic line, HL-60, and have investigated the role of acylation in this process. We used myristic acid as a radioactive precursor, because of the observations cited above. When HL-60 cells are treated with 12-*O*-tetradecanoyl phorbol 13-acetate (TPA), a phorbol ester, they differentiate into functional monocyte-like cells [9]. However, in order to identify non-specific acylation induced by the TPA, it was necessary to study in parallel an HL-60 cell line resistant to TPA differentiation. It has been well established that TPA affects lipid metabolism [10,11], possibly replacing diglyceride, which is the physiological activator of protein kinase C, a receptor for phorbol ester [12]. Here, we have examined both protein and lipid acylation during the differentiation of HL-60 cells. We show that an ~82 kDa protein becomes specifically acylated during the early steps of differentiation.

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## 2. MATERIALS AND METHODS

### 2.1. *Cell culture and reagents*

HL-60 cells were originally obtained from Dr R. Gallo [13] and HL-60 Blast II cells from Dr P. Major [14]. Cells were grown in a humidified atmosphere of 5% $CO_2$ at 37°C in RPMI 1640 medium and 10% fetal calf serum. TPA was added to the cultures at a concentration of 8 ng per ml. [9,10(n)-$^3$H]Myristic acid (41 Ci/mmol) and L-[U-$^{14}$C]serine (137 mCi/mmol) were purchased from Amersham International.

### 2.2. *Lipid extraction and separation*

After incubation with the radioactive fatty acid, cells were harvested by centrifugation and washed twice with isotonic saline. The lipids were then extracted with chloroform/methanol (2:1) as described [15]. A mixture of unlabelled phospholipids was added as carrier to an aliquot of each extract and the phospholipids were separated by thin-layer chromatography (TLC) on 0.25 mm pre-coated silica gel 60 plates (E, Merck) in the two-dimensional solvent system described by Portoukalian et al. [16]. Phospholipids were located by Dittmer's reagent and labelled phospholipids were scraped into liquid scintillation vials for determination of the radioactivity incorporated.

Neutral lipids were separated by two-dimensional TLC. The lipids were spotted on a 10 × 5 cm silica gel 60 plate along with a standard neutral lipid mixture. The plate was run in the shorter dimension in the solvent system chloroform/methanol (50:3.6, v/v) until the solvent front ascended to 0.5 cm from the top of the plate. The plate was then dried and run in the second dimension with the solvent system heptane/diisopropylic ether/glacial acetic acid (60:40:4, v/v) up to the bottom edge of the plate. Lipids were visualized by spraying a vanillin-sulfuric acid reagent. The plates were heated at 120°C until the colour developed. Areas corresponding to lipids were scraped off and counted in scintillation vials with 3 ml of an ethanol-water (50:50) solution and 9 ml picofluor.

### 2.3. *SDS-polyacrylamide gel electrophoresis*

After delipidation, proteins were denatured in 2% SDS, 5% 2-mercaptoethanol, 0.065 M Tris-HCl (pH 7), 15% glycerol and 0.01% bromophenol blue by boiling for 2 min. Samples were then analyzed by SDS-PAGE on 8% gels according to Laemmli [17]. After electrophoresis, gels were treated with 20% 2,5-diphenyloxazole in dimethyl sulfoxide prior to fluorography. Autoradiography was performed at −70°C using an Agfa-Gevaert curix RP2 film.

## 3. RESULTS AND DISCUSSION

The delipidated proteins of HL-60 and HL-60 Blast II cells, which were labelled for 9 h with [$^3$H]myristic acid in the presence or absence of TPA were examined by one-dimensional polyacrylamide gel electrophoresis (fig.1).

Although the overall patterns of the labelled proteins were similar, differences in the level of acylation of several bands were observed. A pro-

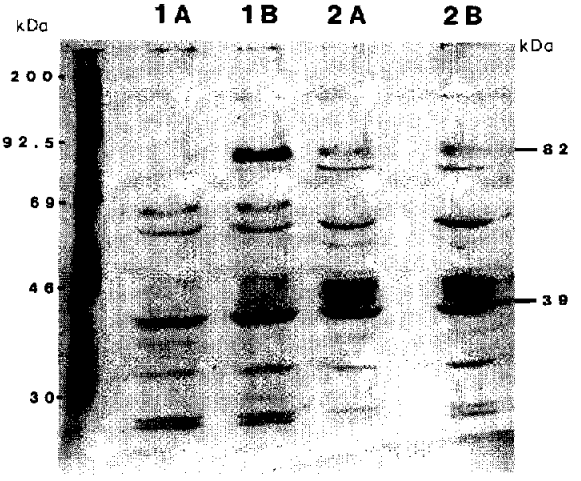


Fig.1. Effect of TPA on the acylation of proteins of TPA-sensitive and TPA-resistant HL-60 cells labelled with [$^3$H]myristate. Exponentially growing cells were incubated with [$^3$H]myristate (11 $\mu$Ci/ml) in 25 cm$^2$ tissue culture flasks in complete medium (5 ml) for 9 h in the absence or presence of TPA. Cells were harvested and after delipidation proteins were analyzed by SDS-PAGE. The gel was calibrated with [$^{14}$C]methylated protein molecular mass markers (left) run in parallel, namely myosin (200 kDa), phosphorylase *b* (92.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa) and lysozyme (14.3 kDa). Gels were exposed for 6 weeks. HL-60 cells either untreated (1A) or TPA-treated (1B), 170 $\mu$g protein/lane. HL-60 Blast II cells either untreated (2A) or TPA-treated (2B), 380 $\mu$g protein/lane.

tein corresponding to a molecular mass of ~63 kDa was present in the sensitive cells and not in the resistant ones, conversely several bands between 40 and 45 kDa were more intensely labelled in the resistant cells.

During differentiation of HL-60 cells with TPA, a major band at 82 kDa was myristylated and several other bands to a lesser extent. The relationship to differentiation was confirmed by comparison with the TPA-resistant cell line (fig.1, lanes 2A,B). As the overall level of acylation was lower in these cells, twice as much cellular protein was loaded onto the gels to compare the spectrum of the protein acylated.

Experiments in which cells were radiolabelled for 23 h with [$^{14}$C]serine (0.33 $\mu$Ci/ml RPMI) and examined by SDS-PAGE showed that the acylated proteins did not correspond to the major protein species of HL-60 cells except for one corresponding to a molecular mass of ~39 kDa (not shown).

Our observations that the radiolabelled myristylated proteins are stable to boiling and extraction with chloroform/methanol confirms that they are covalently linked. To determine the type of linkage, fixed gels were soaked for 3 h in 1 M hydroxylamine-HCl (pH 8), as described in [1] prior to fluorography. All the bands were found to be resistant to hydroxylamine (not shown). When the myristate-labelled proteins were treated for 2 h with 3 N HCl in 65% methanol at 90°C in sealed tubes along with a standard fatty acid mixture and then extracted with diisopropylic ether and the extract analyzed with the two-dimensional TLC system described for neutral lipid analysis (section 2), 93% of the total radioactivity was recovered as fatty acids or esters.

Analysis of the lipids labelled with [$^{3}$H]myristate in the two cell lines, HL-60 and Blast II, in the presence and absence of TPA is given in table 1. In the TPA-sensitive HL-60 cells, only small increases

Table 1

Effect of TPA on the acylation of lipids of TPA-sensitive and TPA-resistant HL-60 cells labelled with [$^{3}$H]myristate

| Lipids | cpm/$\mu$g protein | | | |
|---|---|---|---|---|
| | HL-60 | | HL-60 Blast II | |
| | Control | TPA | Control | TPA |
| Monoglyceride | 112 ± 5 | 81 ± 5 | 68 ± 7 | 78 ± 5 |
| 1,2-Diglyceride | 413 ± 30 | 435 ± 22 | 237 ± 19 | 240 ± 10 |
| 1,3-Diglyceride | 158 ± 9 | 200 ± 10 | 207 ± 13 | 240 ± 14 |
| Cholesterol | 42 ± 5 | 48 ± 7 | 62 ± 8 | 75 ± 8 |
| Lanosterol | 51 ± 6 | 62 ± 4 | 56 ± 3 | 117 ± 9 |
| Fatty acid | 76 ± 8 | 54 ± 5 | 32 ± 3 | 27 ± 3 |
| Triglyceride | 2592 ± 130 | 2587 ± 138 | 1299 ± 65 | 1274 ± 71 |
| Fatty acid ethyl ester | 222 ± 13 | 356 ± 19 | 543 ± 32 | 871 ± 37 |
| Cholesterol ester | 68 ± 6 | 33 ± 5 | 132 ± 8 | 97 ± 7 |
| Lysophosphatidylcholine | 45 ± 4 | 75 ± 5 | 26 ± 3 | 23 ± 4 |
| Sphingomyelin | 241 ± 14 | 409 ± 19 | 84 ± 5 | 110 ± 7 |
| Phosphatidylcholine | 16063 ± 700 | 17449 ± 871 | 7308 ± 335 | 6975 ± 31 |
| Phosphatidic acid | 301 ± 17 | 281 ± 22 | 90 ± 4 | 64 ± 4 |
| Lysophosphatidylethanolamine | 72 ± 4 | 77 ± 6 | 30 ± 2 | 21 ± 2 |
| Phosphatidylinositol | 599 ± 36 | 578 ± 33 | 192 ± 11 | 177 ± 10 |
| Phosphatidylserine | 315 ± 14 | 328 ± 18 | 132 ± 7 | 135 ± 6 |
| Phosphatidylethanolamine | 1125 ± 46 | 1210 ± 57 | 356 ± 22 | 357 ± 18 |

After incubation with [$^{3}$H]myristate (see legend to fig.1) cells were extracted and the lipids were analysed as indicated in section 2. Results are the average ± SD of two determinations of one of 3 experiments which gave similar results

were detected in incorporation of radioactivity into 1,3-diglyceride, fatty acid ethyl ester, lysophosphatidylcholine and sphingomyelin after treatment with TPA, but a decrease in incorporation was observed in monoglyceride, free fatty acid and cholesterol ester.

The incorporation of [$^3$H]myristic acid into lipids of the TPA-resistant cells was also modified by TPA treatment. Although the total incorporation was less than half that of the TPA-sensitive cells, TPA increased incorporation into sphingomyelin, lanosterol and fatty acid esters compared to untreated cells. The distribution of [$^3$H]myristate among the various lipids of HL-60 and Blast II cells differed and corresponded to the differences observed in their lipid composition (Malvoisin, E., unpublished).

Our studies have shown that TPA induction of differentiation in HL-60 cells is accompanied by induction of the myristylation of a protein at ~82 kDa and to a lesser extent a number of other proteins. This event occurs early in the differentiation process, before the irreversible step (~16 h) [10] and was not found in cells resistant to TPA differentiation.

The changes observed in the lipid fractions during differentiation are more difficult to interpret, as changes were found in both sensitive and resistant cells. However, certain changes could be associated with differentiation since they are absent or less affected in the TPA-resistant cells. The enhancement of protein myristylation by TPA in HL-60 cells appears more specific than that of protein phosphorylation, in which at least 14 proteins are involved [18–23]. By comparison with other cells where myristylated proteins are present in the cytosol [1], we suggest that phosphorylation and myristylation may be complementary events. Thus, a cytosolic phosphorylated protein can be translocated to the membrane by myristylation, the covalently bound fatty acid serving as an anchor to bind the soluble protein to the membrane. It is likely that TPA-induced myristylation of cellular polypeptides plays a primary role in the differentiation process.

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