Acylation *in vivo* of the contact site A glycoprotein and of other membrane proteins in *Dictyostelium discoideum*

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Cells of *Dictyostelium discoideum* were incubated during the preaggregation phase with [^{3}H]palmitic acid, and association of the fatty acid label with proteins was investigated by SDS-polyacrylamide gel electrophoresis and fluorography. Three proteins with apparent molecular masses of 44, 80 and 130 kDa were preferentially labeled. The 80-kDa protein proved to be identical with an integral membrane glycoprotein called contact site A. The protein retained the ^{3}H label after chloroform-methanol extraction and boiling in SDS-containing buffer, but not after mild hydroxylamine treatment, indicating an ester bond. After treatment of the protein with KOH the ^{3}H label was recovered in free palmitic acid, as demonstrated by gas-liquid chromatography of the methyl ester.

Acylation *Integral membrane glycoprotein* *Cell adhesion* Dictyostelium discoideum

1. INTRODUCTION

When cells of *Dictyostelium discoideum* acquire the aggregation-competent stage, they express on their surfaces a particular glycoprotein with an apparent molecular mass of 80 kDa [1]. This glycoprotein has been discovered as an antigen that binds adhesion-blocking Fab species from antisera raised against whole membrane proteins of aggregation-competent cells [1–3]. The Fab species which are neutralized by the 80-kDa glycoprotein block the EDTA-stable type of cell adhesion of aggregation-component cells. This type of intercellular adhesion is not detected in growth-phase cells. Because of their possible involvement in cell adhesion the developmentally regulated target sites of the adhesion-blocking Fab, which are specific for the aggregation-component stage, have been called contact sites A [1].

The contact site A glycoprotein is distinguished from other glycoproteins of *D.discoideum* by a variety of post-translational modifications. The protein is phosphorylated at serine residues [4,5], and the carbohydrate moieties are extensively sulfated [6]. Here we show that the 80-kDa glycoprotein is acylated by palmitic acid, and that two other membrane proteins are also prominently labeled after incubation of developing cells with [^{3}H]palmitic acid.

2. METHODS

2.1. Cell culture and in vivo labeling with [^{3}H]palmitic acid, [^{35}S]methionine and [^{35}S]sulfate

Cells of *D.discoideum* strain AX2-214 were grown axenically as in [7], harvested at a density of 5×10^6 cells/ml, washed free of nutrient, and adjusted to 1×10^7 cells/ml in 17 mM Soerensen phosphate buffer (pH 6.0). The starving cells were shaken in suspension for 2 h. For labeling with palmitic acid, 2 ml suspension were transferred to a screw-cap tube in which 2–5 mCi [^{3}H]palmitic acid (NEN, 23.5 Ci/mmol) had been taken up by sonication in 100 μl of a solution containing 100 mM sodium pyruvate and 100 mM $MgCl_2$ [8,9]. The cells were incubated for 3 h under shaking at 250 rpm. For labeling with methionine, cells were incubated for 4 h with 0.3 mCi [^{35}S]methionine/ml (Amersham, 800 Ci/mmol). Labeling with carrier-free [^{35}S]sulfate (0.3 mCi per ml; SJS 1, Amersham) was performed as in [6]. All experiments with living cells were done at 23°C. Crude particulate fractions

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were prepared by freeze-thawing of washed cells and centrifuged for 20 min at 10000 × *g*.

2.2. Gel electrophoresis and immunoblotting

SDS-polyacrylamide electrophoresis (SDS–PAGE) gels [10], two-dimensional electrophoresis [11] and fluorography with PPO [12,13] were standard methods. For transfer to nitrocellulose (BA 85, Schleicher and Schüll) the method of [14] was used as described [15,16]. For immunolabeling of blots after fluorography, PPO was washed off the nitrocellulose filter with toluene. After evaporation of toluene the filter was incubated with ^{125}I-labeled mAb 20-6-4. The IgG of this monoclonal antibody raised against the 80-kDa glycoprotein [17] belonged to subclass 2A as tested with subclass specific antisera from Meloy, Springfield, VA.

2.3. Hydroxylamine treatment

After SDS–PAGE of a particular fraction from [^{3}H]palmitic acid-labeled cells, one lane was fixed and treated for 2 h at room temperature at pH 6.6 with 1 M hydroxylamine as in [18], and was fluorographed together with a second lane which served as a control.

2.4. Identification of protein-bound fatty acid

Particular fractions were extracted once with a 1:1 mixture and twice with a 2:1 (v/v) mixture of chloroform and methanol at protein concentrations below 1 mg/ml [19]. After SDS–PAGE and fluorography tritiated bands were excised from the gel, saponified for 1.5 h at 40°C with 0.6 M KOH in 96% ethanol. After acidification with HCl, chloroform and water were added to yield an 8:4:3 ratio of chloroform/ethanol/water. Fatty acids were recovered from the lower phase and converted to methyl esters by diazomethane in diethyl ether.

The ^{3}H-labeled methylated compounds were separated isothermally by gas liquid chromatography on an OV 1 column at 190°C. The gas effluent was fractionated by trapping on silicone-coated glass beads using a Packard model 851 fraction collector [20,21] and analysed in a liquid scintillation counter.

3. RESULTS

The incorporation of palmitic acid into proteins was investigated during the preaggregation phase of *D.discoideum*, a period during which growth-phase cells acquire the ability to aggregate. At 5 h of starvation a marker of aggregation-competent cells, the 80-kDa glycoprotein called contact site A, was clearly detectable by labeling with a specific monoclonal antibody, mAb 20-6-4 [6,17]. At this time the cells were homogenized. The particulate fraction was extracted as in [19] to remove free palmitic acid together with the majority of cellular lipids. The residue was boiled in sample buffer containing 2% SDS and subjected to SDS–PAGE followed by fluorography.

The fluorograms showed 3 prominently labeled bands which corresponded to proteins with molecular masses of 44, 80 and 130 kDa. In addition, about 30 faintly labeled bands were detectable, as well as a broad smear of labeled material (fig.1C). The linkage of the ^{3}H-labeled material to the proteins was sensitive to mild hydroxylamine treatment. After incubation of one lane of the gel with hydroxylamine no ^{3}H label remained detectable at the positions of the protein bands (fig.1D).

Incorporation of [^{3}H]palmitic acid into polypeptides did not correlate with [^{35}S]methionine labeling during the early period of development (fig.1A). This result indicates that among the de novo synthesized proteins associated with the particulate fraction of cells, only a few selectively received the [^{3}H]palmitic acid.

Glycoproteins have been labeled with [^{35}S]sulfate during early development, and two prominently labeled bands with apparent molecular mases of 80 and 130-kDa have been detected [6]. As shown in fig.1B,C these strongly sulfate-labeled bands coincided with two of the bands most heavily labeled with [^{3}H]palmitic acid.

The sulfated 80-kDa protein has been identified as the contact site A glycoprotein [6]. To confirm that the label of [^{3}H]palmitic acid was associated with this particular glycoprotein, proteins were separated by two-dimensional electrophoresis and blotted onto nitrocellulose. On fluorograms a series of spots was seen in the 80-kDa position, centered at pH 4.8, typical of the contact site A glycoprotein (fig.2, top). After fluorography the blots were incubated with iodinated mAb 20-6-4, and specifically the spots in the 80-kDa position were labeled. All the spots labeled with the antibody were also detected on the fluorograms, indi-

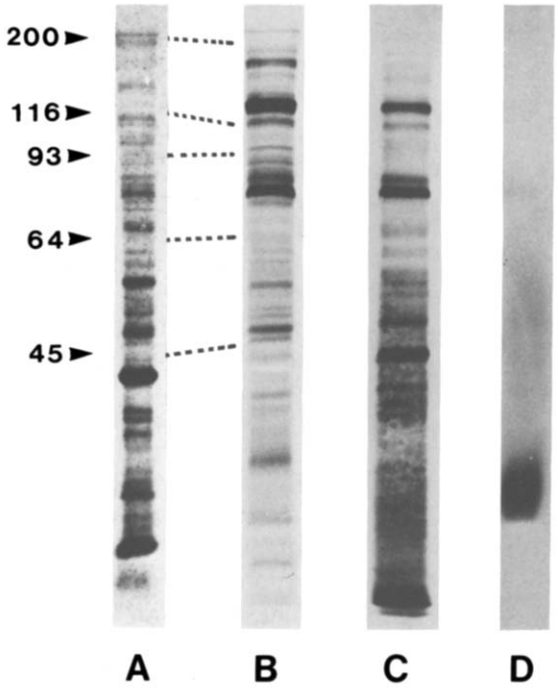

Fig.1. Autoradiograms obtained from particulate fractions of cells labeled with (A) [^{35}S]methionine, (B) [^{35}S]sulfate, and (C,D) [^{3}H]palmitic acid. (A–C) Proteins separated by SDS–PAGE were blotted onto nitrocellulose, and the filter was autoradiographed without enhancer (A,B) or fluorographed with PPO (C). (D) The gel was treated with 1 M hydroxylamine at pH 6.6 and directly fluorographed. To cells starved for 2 h in suspension, the radiolabeled compounds were added. After 3 h incubation, or 4 h in (A), particulate fractions were prepared from the cells. The pattern of [^{35}S]methionine-labeled polypeptides (A) provides a basis for comparing the number of strongly [^{3}H]palmitic acid-labeled bands (C) with the total number of polypeptides associated with the particulate fraction and synthesized during the early phase of development. None of the 3 bands most strongly labeled with palmitic acid is seen as a prominent band after [^{35}S]methionine labeling. Values on the left indicate molecular mass in kDa.

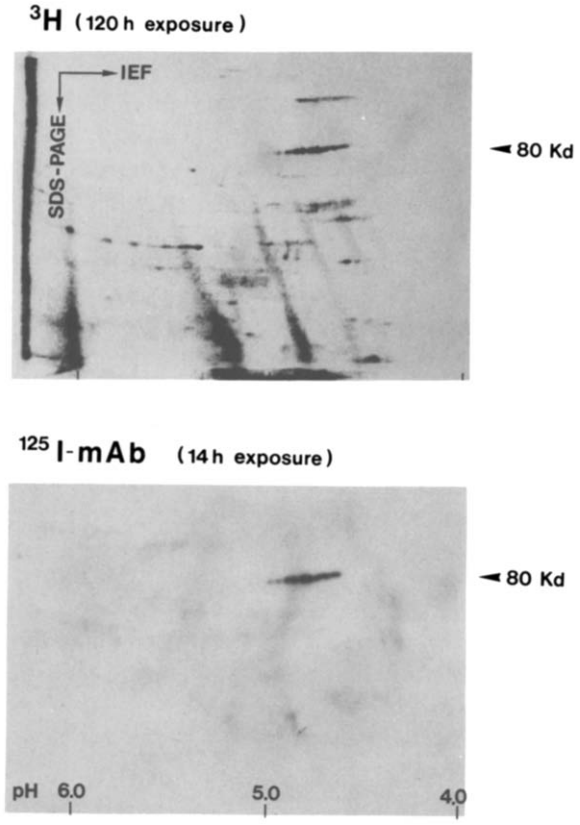

Fig.2. Two-dimensional electrophoresis of proteins from the particulate fraction of cells incubated with [^{3}H]palmitic acid (top), and identification of the contact site A glycoprotein by monoclonal antibody (bottom). An aliquot of the same SDS-solubilized fraction as in fig.1C was concentrated by precipitation with acetone (9:1, v/v), the proteins subjected to isoelectrofocusing and SDS-electrophoresis in 10% polyacrylamide gel, and blotted onto a nitrocellulose filter. (Top) The filter was fluorographed with an exposure time of 120 h. (Bottom) The same filter was incubated, after removal of the enhancer, with ^{125}I-labeled mAb 20-6-4 which is specific for the contact site A glycoprotein [17], and was exposed for 14 h.

cating that all isoelectric variants carried the ^{3}H label from palmitic acid (fig.2, bottom).

The [^{3}H]palmitic acid-labeled 130-kDa material formed a number of spots within the same pH range as the 80-kDa glycoprotein. The non-sulfated 44-kDa material proved to be less acidic, but also consisted of a number of [^{3}H]palmitic acid-labeled isoelectric variants, which were distributed over a large interval between pH 5.0 and 6.0. (It is possible that some variants were lost at the alkaline side of the gel.) The smear of labeled material observed after one-dimensional electrophoresis formed, after two-dimensional separation, 3 diagonal bands typical of lipids (fig.2, top). These lipids have apparently resisted chloroform–methanol extraction, as is known to be the case for glycosphingolipids of *D.discoideum* [22].

To identify the radioactive compound linked to

proteins in [^{3}H]palmitic acid-labeled cells, non-covalently associated ^{3}H-labeled compounds were exhaustively extracted from proteins of the particulate fraction with chloroform–methanol [19]. The radioactivity dissolved after the third extraction in the soluble fraction was less than 10% of the label that remained in the proteinaceous residue. The residue was subjected to SDS–PAGE. After fluorography the 80 and 130-kDa bands were excised from the gel. The fatty acids liberated by alkaline hydrolysis were methylated and separated by gas-liquid chromatography. The major peak of radioactivity obtained from either the 130- or 80-kDa band coincided with the peak of authentic palmitic acid methyl ester (fig.3). Two minor peaks were observed which might indicate partial conversion in vivo of the palmitic acid into other fatty acids.

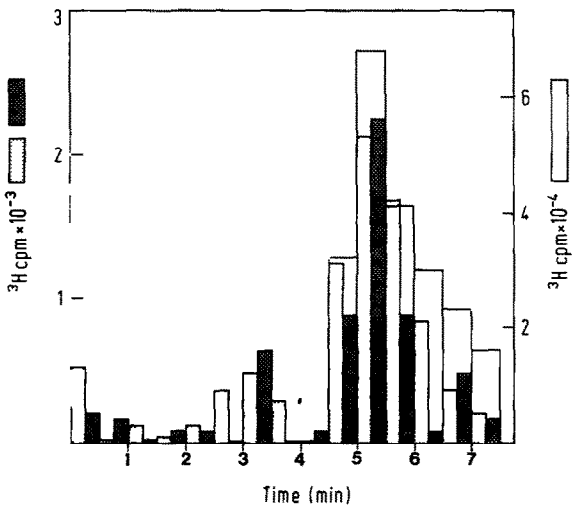

Fig.3. Gas/liquid chromatography of the [^{3}H]palmitic acid-labeled compounds cleaved from the 80-kDa glycoprotein (lightly shaded) and from the 130-kDa glycoproteins (heavily shaded) by ethanolic KOH, in comparison to authentic [^{3}H]palmitic acid (unshaded columns). The samples were methylated with diazomethane, the methyl esters separated on an OV 1 column trapped on silicone-coated glass beads changed at 0.5-min intervals, and the radioactivity was determined by liquid scintillation counting. The protein bands were sliced out from polyacrylamide gels. Background of radioactivity, about 200 cpm, was subtracted. The retention time of the [^{3}H]palmitic acid peak coincided with that of unlabeled palmitic acid methyl ester detected by flame ionisation.

4. DISCUSSION

After discovery of the lipoprotein nature of an outer membrane protein of *E.coli* [23], acylation has been demonstrated for vesicular stomatitis virus glycoprotein [24] and subsequently for a number of other membrane proteins in viruses, procaryotes and eucaryotic cells (review [25]). The large tumor specific antigen in SV40 infected cells exists in the nucleus in a non-acylated state, and in the plasma membrane in an acylated state [26]. In trypanosomes the variant glycoproteins are acylated when they are associated with the cell surface, and are shed from the cells after deacylation [27].

The 80-kDa glycoprotein of *D.discoideum* shown to be a lipoprotein falls in the same category as the other known acylated proteins from eucaryotic cells or animal viruses, which are all glycosylated, integral membrane proteins. The acylated 130-kDa protein has been shown to be developmentally regulated and sulfated at carbohydrate residues, as is the 80-kDa glycoprotein [6]. The third strongly acylated membrane protein of developing cells cannot be identified with another known membrane protein of *D.discoideum;* its apparent molecular mass of 44 kDa is similar to that of proteins previously suggested to represent cyclic AMP receptors inolved in the chemotactic response [28,29].

The 80-kDa glycoprotein has been purified as a target site of univalent antibody fragments (Fab) that block the EDTA-stable type of cell–cell contact. The glycoprotein, also called contact site A, becomes detectable at the late preaggregation phase, when the cells begin to form EDTA-stable contacts. Typically, aggregating cells are elongated, and the EDTA-stable contacts are formed at the ends of the cells. Immunolabeling showed that, at least at the beginning of aggregation, the contact site A glycoprotein is distributed over the whole cell surface, probably clustered into oligomers [15]. It is possible that the glycoprotein can interact with the cytoskeleton, and that it is activated by this interaction specifically at the ends of the cells. The preferential labeling of the protein with [^{3}H]palmitic acid suggests that the fatty acid is important for one of these activities, although the functional consequence of the acylation cannot be specified as yet.

There are at least two other developmentally

regulated membrane proteins of aggregating *D.discoideum* cells which are difficult to solubilize with detergents: cyclic AMP receptors at the cell surface and adenylate cyclase. Because they are not extracted with 1% Triton X-100 the cyclic AMP receptors have been proposed to be associated with the cytoskeleton [30]. Proteins labeled by [^{3}H]palmitic acid are enriched in the insoluble material after extraction of a particulate fraction with 1% Triton X-100 (J. Hagmann, unpublished). It is conceivable that these proteins are difficult to solubilize because they are lipoproteins.

Cyclic AMP phosphodiesterase is released by *D.discoideum* cells into the extracellular space and is also associated with the cell surfaces. In a mutant, HPX 235, both the released and membrane-bound phosphodiesterases are inactive or down-regulated [31]. The membrane-bound phosphodiesterase appears to have a hydrophobic site which is missing in the soluble phosphodiesterase [32]. It would be of interest to discover whether these phosphodiesterases differ primarily by the presence of fatty acid in the membrane-bound form.

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