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AN ANALYSIS OF THE PROTEIN, GLYCOPROTEIN AND MONOSACCHARIDE COMPOSITION OF *DICTYOSTELIUM DISCOIDEUM* PLASMA MEMBRANES DURING DEVELOPMENT

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Summary

Qualitative and quantitative changes in the protein and glycoprotein components of the plasma membrane of the cellular slime mould *Dictyostelium discoideum* have been detected by analysis of sodium dodecyl sulphate-polyacrylamide gel electrophoretic patterns. The amounts of proteins of subunit molecular weight 220 000, 91 000, 63 000, 59 000, 58 000, 56 000 increased during the acquisition of aggregation competence, while proteins of subunit molecular weight 82 000 and 22 000 decreased. The amounts of glycoproteins with apparent subunit molecular weights 285 000, 150 000, 137 000, 100 000, 53 000, 50 500 and 30 500 increased during differentiation while a 125 000 dalton component decreased dramatically in amount. The neutral and amino sugar composition of the plasma membrane was also analyzed and found to remain essentially unchanged during the first 12 h of differentiation. The major sugars were mannose, fucose, and glucosamine; galactose and galactosamine were also present, but in lower amounts.

Introduction

Two distinct phases are recognizable in the life cycle of the cellular slime mould *Dictyostelium discoideum*: a vegetative phase in which unicellular amoebae exist independently and multiply by binary fission, and a multi-

Abbreviation: SDS, sodium dodecyl sulphate.

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cellular phase characterized by the formation of stable, species specific cell-cell contacts and by the subsequent differentiation into a fruiting body comprising two distinct cell types [1]. The acquisition of aggregation competence, triggered by nutrient depletion and detectable 8—9 h later [2], is accompanied by several molecular events at the cell surface. These include the appearance of a new class of surface antigens, blocking of which by univalent antibodies prevents aggregation [2—4], and changes in exposed surface proteins, detected by electrophoretic analysis of ^{125}I-labelled whole cells [5,6].

Proteins have been implicated in the aggregation process since trypsin inhibits aggregate formation [7]. Furthermore, the appearance of a carbohydrate-binding protein, discoidin, at the cell surface accompanies the acquisition of aggregation competence, and it has been suggested that this molecule is involved in a carbohydrate-protein interaction required for adhesion [8,9]. There have also been several reports documenting changes in lectin agglutination and binding during the transition from growth to aggregation [10—14], suggesting possible modifications of the cell surface carbohydrates.

It is important to analyze the developmentally regulated modifications in the protein and glycoprotein components and in the carbohydrate moieties. There have been several analyses of the plasma membrane components of *D. discoideum* strains A3 and NC4 [56,15,16] and it has been claimed that a number of components are developmentally regulated. There is little agreement, however, with regard to which components are regulated, despite the fact that both strains undergo a similar developmental program. It is possible, therefore, that some of the membrane changes that have been described have little relevance to the developmental process.

We have analyzed the plasma membrane components of a third strain, Ax-2, to see if any of the previously reported developmentally regulated alterations are detectable in this strain. Any changes in the components of plasma membrane that are vitally important for development should be apparent in all strains. In addition we have analyzed the carbohydrate content of the plasma membranes at various stages of development. The results of these studies are presented in this report.

Materials and Methods

Materials. Bacteriological peptone and yeast extract were obtained from Oxoid Ltd. Reagents for gel electrophoresis were obtained from Bio-Rad Laboratories. Materials for plasma membrane preparation and gas-liquid chromatography were obtained as previously listed [18]. Other reagents were the best available grade from Fisher Scientific Co. or Sigma Chemical Co.

Organism and growth conditions. An axenic mutant (strain Ax-2) of *D. discoideum* [17], obtained from Dr. J.M. Ashworth was used throughout these studies. The strain was grown on HL5 medium previously described [10], to a density of $5—8 \cdot 10^6$ cells/ml. At this stage amoebae were harvested as vegetative (0 h) cells by centrifugation at $700 \times g$ for 3 min.

To obtain cells at various stages of the aggregation process, vegetative cells were washed and resuspended to the same density in 16.7 mM potassium phosphate/disodium phosphate, pH 6.0 and shaken for the appropriate period

(4 h, 8 h or 12 h), as described by Beug et al. [3].

Preparation of plasma membranes and gel electrophoresis. Harvested cells were washed and membranes prepared as previously described [18,19]. PM1 and PM2 membrane fractions were retained for subsequent analysis.

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis slabs were prepared and run essentially as described by Studier [20]. The acrylamide concentration in the stacking and separating gels was 3% and 10%, respectively. Plasma membrane preparations were resuspended in sample buffer [20] at a concentration of 5—10 mg/ml and the SDS concentration adjusted to 3 mg/mg protein. Samples were routinely heated for 2 min at 100°C and bromophenol blue added to a final concentration of 0.001% as a tracking dye. Aliquots (75 μg protein for eventual protein staining, or 250 μg protein for carbohydrate staining) were loaded in 4-mm wide wells in 2-mm thick gels and the samples electrophoresed for 30 min at 5 mA followed by approximately 3 h at 25 mA. A calibration of molecular weight vs. migration distance was obtained by electrophoresis of the following proteins of known molecular weight: myosin (220 000), β-galactosidase (130 000), phosphorylase *B* (90 000), bovine serum albumin (65 000), ovalbumin (45 000), chymotrypsinogen (25 000) and cytochrome *c* (12 400).

Following gel electrophoresis some gels were fixed and stained for protein with 0.2% Coomassie Blue in a solution of methanol/acetic acid/H_2O (5 : 1 :5) and were destained in methanol/acetic acid/H_2O (5 : 1 : 5). Gels were immersed in acetic acid/H_2O (1 : 9) 30 min prior to being scanned or photographed. Other gels were fixed and stained for carbohydrate using a procedure modified from the periodic acid-Schiff's staining procedure described by Fairbanks et al. [21]. Gels were fixed in 25% isopropanol, 10% acetic acid for 12 h, with two changes of the fixing solution during that period. Oxidation was achieved by immersion in 0.5% periodic acid for 2 h at 4°C in the dark. Gels were rinsed with 0.5% sodium arsenite, 5% acetic acid for 45 min and stained with freshly prepared Schiff's reagent [21] for 2 h at 4°C in the dark. Finally gels were washed with 0.1% sodium metabisulfite, 0.01 N HCl until the background was clear. Stained gels were scanned with an adapted Quick-Scan densitometer/integrator (Helena Laboratories, Beaumont, Texas), slit-width 0.16 × 16 mm, at 610 and 525 nm for protein and carbohydrate, respectively.

Carbohydrate analyses. Membrane-bound neutral sugars were analyzed by gas-liquid chromatography of their alditol acetate derivatives by a procedure modified from that given by Albersheim et al. [22]. Membrane samples were dialyzed using Spectropore 3 tubing for 24 h at 0°C against three changes of distilled water and lyophilized. The lyophilized samples (2—3 mg protein) were hydrolyzed for 1 h with 0.5 ml of 1 N trifluoroacetic acid at 121°C, in screw-cap tubes. After hydrolysis the soluble portion was evaporated to dryness and reduced with $NaBH_4$ as described by Albersheim et al. [22]. Samples were acetylated with 0.5 ml acetic anhydride for 1 h at 100°C, concentrated to near dryness under N_2 and redissolved in a small volume of chloroform for chromatographic analysis. Gas-liquid chromatography was done using a 3% ECNSS-M column as previously described [18].

Amino sugars were analyzed on the short column of a Beckman automatic

amino acid analyzer after hydrolysis of the dialyzed sample (1—2 mg protein) with *p*-toluenesulphonic acid, as described by Lui [23].

Protein was determined by the procedure described by Lowry and coworkers [24].

Results

In order to determine the optimal incubation period for complete dissociation and solubilization of the plasma membrane components, samples were heated at 100°C for various times in sample buffer and SDS, prior to SDS-polyacrylamide gel electrophoresis. The results of this experiment are presented in Fig. 1. It is evident that heating at this temperature for longer than 5 min causes a considerable amount of Coomassie Blue stainable material to remain slightly below the interface between the stacking and running gel during electrophoresis, presumably as a result of protein subunit association. The coincident decrease of a protein of approximate molecular weight 98 000 (p98) * and to a lesser extent a p91 component, suggests that these proteins are responsible for the major part of the material that appears just below the interface.

In view of this result, samples were routinely heated for 2 min in sample buffer to provide maximum dissociation. Under these conditions essentially all the sample applied to the sample-well subsequently entered the running gel during electrophoresis as determined by Coomassie Blue staining. Periodic acid-Schiff staining, however, reveals the presence of material which remains at the interface between the stacking and running gel (Fig. 5), even after heating for 2 min in the presence of SDS, suggesting that certain glycoproteins either have subunits which fail to dissociate or have large subunits. Alternatively, this glycoprotein material may have a large carbohydrate : protein ratio and hence low charge : mass ratio, resulting in a low electrophoretic mobility [25]. Periodic acid-Schiff stained gels of *D. discoideum* plasma membranes presented by other workers also indicate the presence of a slow migrating glycoprotein component [16].

The electrophoretic analyses (Figs. 1—3) resolved approximately 40 distinct protein components in the plasma membrane. A number of minor components are revealed only when gels are densitometrically scanned (compare Figs. 3 and 4). Several plasma membrane preparations have been analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining and identical protein profiles are observed in all cases. Three different preparations are shown in the gels presented in Figs. 1—4, indicating the reproducibility of the preparative and analytical techniques. Hoffman and McMahon [16] reported increased resolution of *D. discoideum* plasma membrane proteins using exponential gradient gels. In contrast, we do not see improved resolution over that shown in Figs. 1—4 using exponential gradients.

The similarity in the Coomassie Blue stained components of our PM1 and PM2 fractions (Fig. 2) should be noted. Previously published data indicated

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* The protein and glycoprotein components have been designated 'p' and 'gp' values respectively, that correspond to their molecular masses (daltons $\times 10^{-3}$).

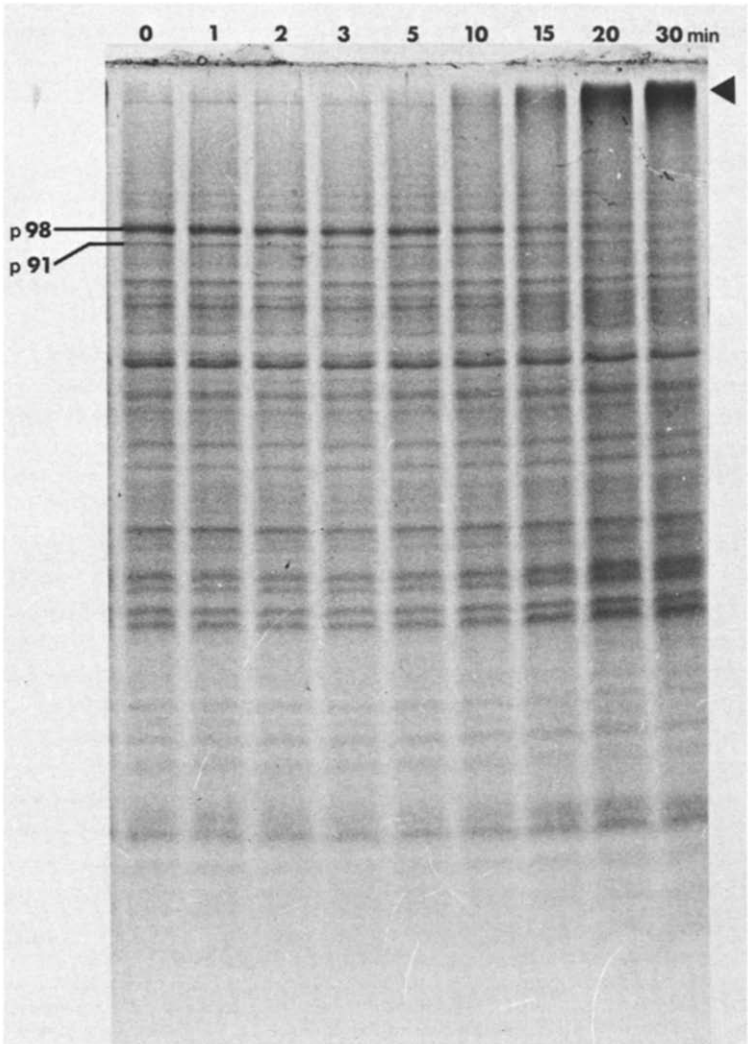

Fig. 1. Effect of preheating in SDS on the dissociation of plasma membrane protein subunits. Plasma membranes were heated in sample buffer [19] at 100°C for the indicated time periods and then electrophoresed as described under Materials and Methods. Gels were stained with Coomassie Blue. The subunit components which disappear on prolonged heating are indicated by their p values and the high molecular weight component that appears is indicated by the arrow (▲).

that the PM2 fraction contained twice as much internal membrane contamination as the PM1 fraction [18]. The fact that the additional contaminating proteins are not discernible by SDS-polyacrylamide gel electrophoresis confirms our conclusion that there is only a low level of contamination in our plasma membrane preparation. The increased amount of certain proteins during aggregation is also unlikely to be an artifact due to contamination since the purity of the aggregation phase membranes is identical to those of the vegetative phase (unpublished observations).

Comparison of the plasma membranes of vegetative and aggregation phase

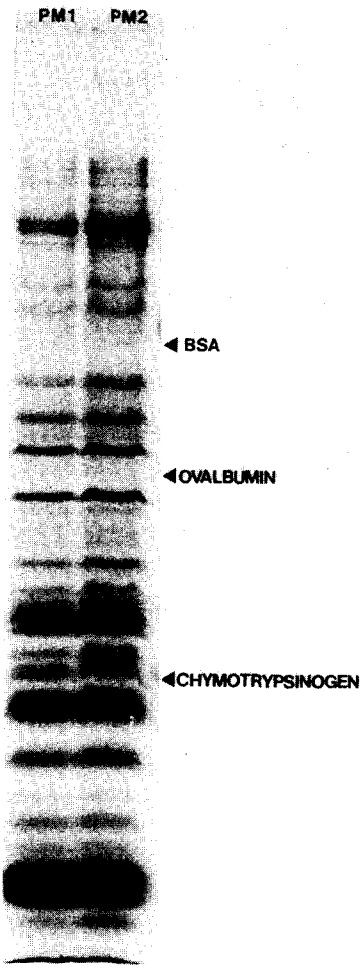

Fig. 2. A comparison of the SDS-polyacrylamide gel electrophoresis patterns of plasma membrane fractions PM1 and PM2. The PM1 and PM2 fractions of a plasma membrane preparation were electrophoresed and stained with Coomassie Blue as described under Materials and Methods. BSA, bovine serum albumin.

cells by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining is shown in Figs. 3 and 4. It is clear that the overall picture is one of conservation rather than drastic modification, although a small number of qualitative and quantitative changes are evident. Only components that undergo an unmistakable change in amount have been denoted in Fig. 4; all of these modifications have been confirmed in three independent experiments. Major increases are seen for components p220, p91, p63, p59, p58, p56, p16 and p15.5 while decreases occur in components p82 and p22. Many of the modifications occur during the first 4 h following the transfer of cells from growth medium to phosphate buffer and are conserved during the remainder of the experimental period. Some additional slight quantitative changes during aggregation are visible in Figs. 3 and 4 (e.g. p100 and p96) but were not consistently observed in replicate experiments.

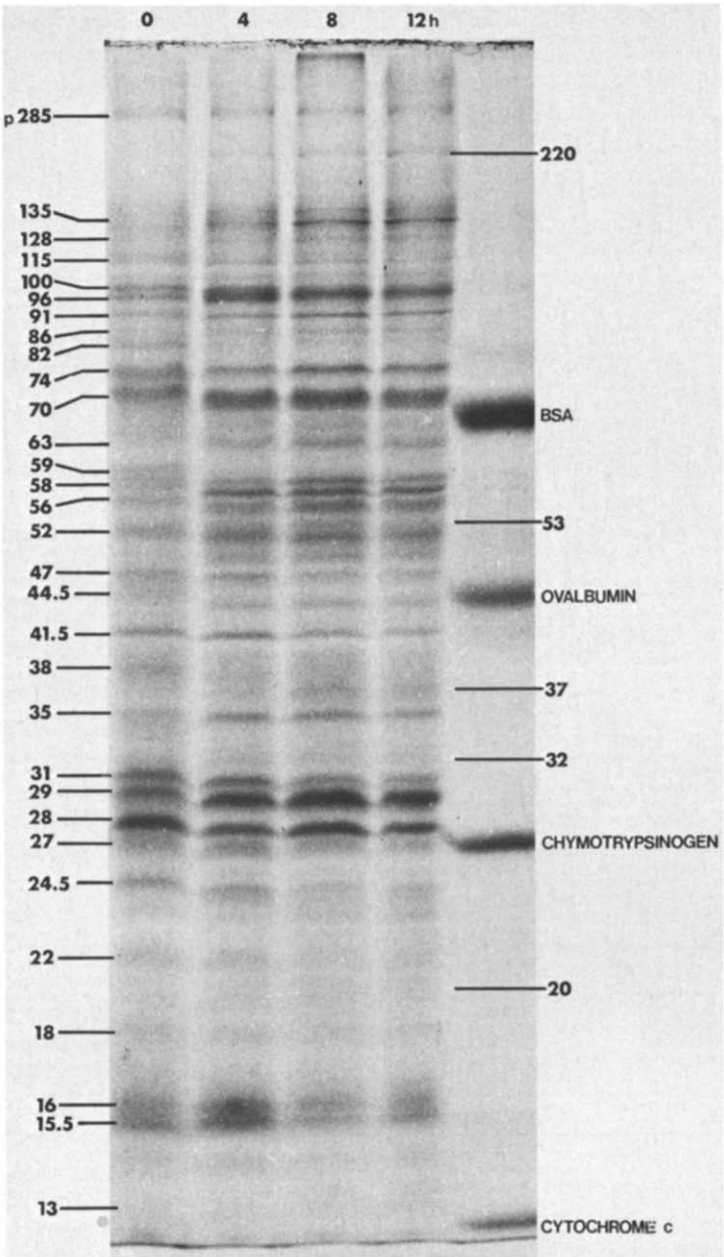

Fig. 3. SDS-polyacrylamide gel electrophoresis of plasma membrane fraction PM1 derived from vegetative (0 h) and aggregating (4, 8 and 12 h) amoebae. Gels were stained for protein with Coomassie Blue. Principle protein components are indicated by their approximate molecular weight $\times 10^{-3}$. Four molecular weight markers are also shown.

The mobility of several proteins appears to increase slightly during the first 4 h of aggregation and this is particularly apparent for three components, p59, p58 and p56. The overall profile presented by this group in densitometric scans is retained suggesting a slight post-translational modification rather than a

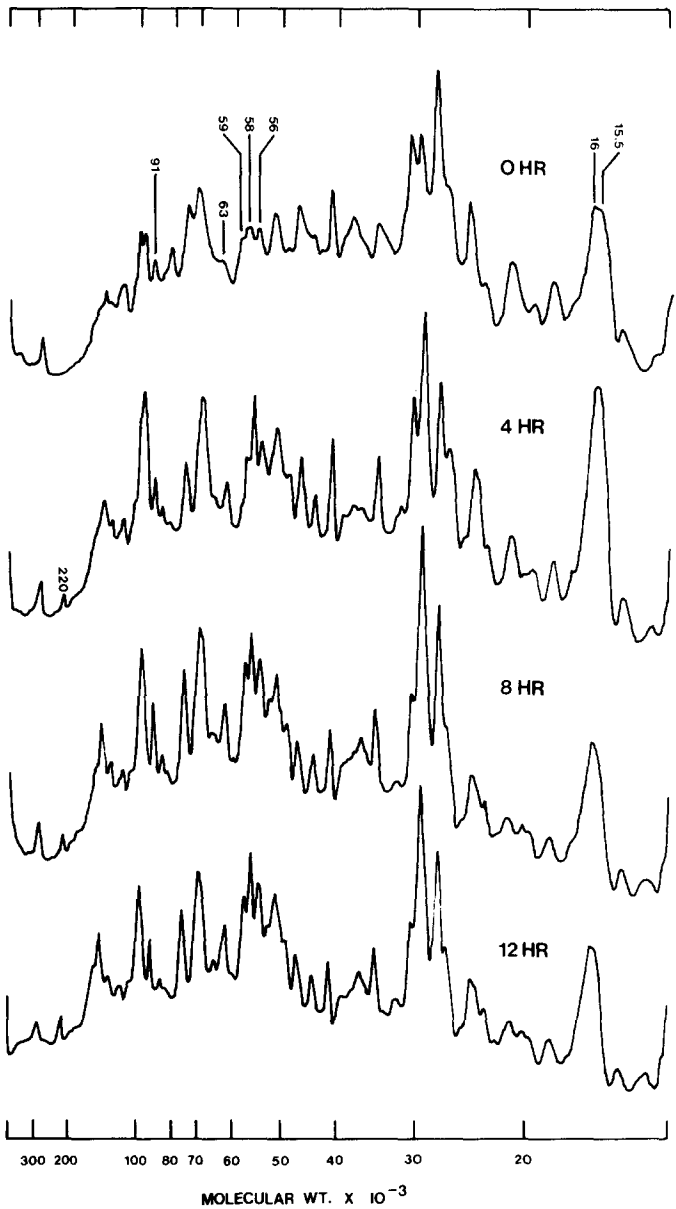

Fig. 4. Densitometric scans of the Coomassie Blue stained gel shown in Fig. 4. Protein components showing significant developmental changes are indicated by their approximate molecular weight X 10^{-3}.

replacement of vegetative components by newly synthesized aggregation components.

Separation of plasma membrane carbohydrate-containing proteins at various stages of differentiation, determined by SDS-polyacrylamide gel electrophoresis and periodic acid-Schiff staining, is presented in Figs. 5 and 6. Approximately 20 glycoprotein components ranging in molecular weight from 285 000 to 22 000 are visible. The broad nature of the stained bands may indicate micro-

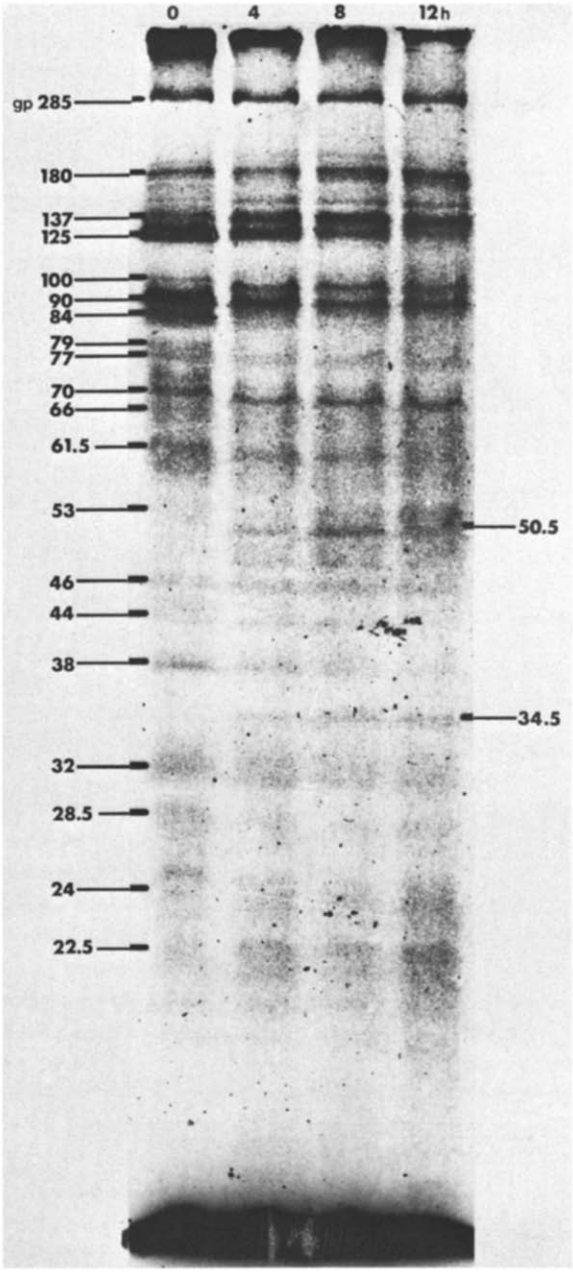

Fig. 5. SDS-polyacrylamide gel electrophoresis of plasma membrane fraction PM1 derived from vegetative (0 h) and aggregating (4, 8 and 12 h) amoebae. Gels were stained for carbohydrate with periodic acid-Schiff reagent. Principle glycoprotein components are indicated by their approximate molecular weight X 10^{-3}.

heterogeneity of the carbohydrate moiety of the glycoproteins [26] or merely reflect the large loadings of membrane protein necessitated by the insensitivity of periodic acid-Schiff staining. Since not all glycoproteins stain with periodic

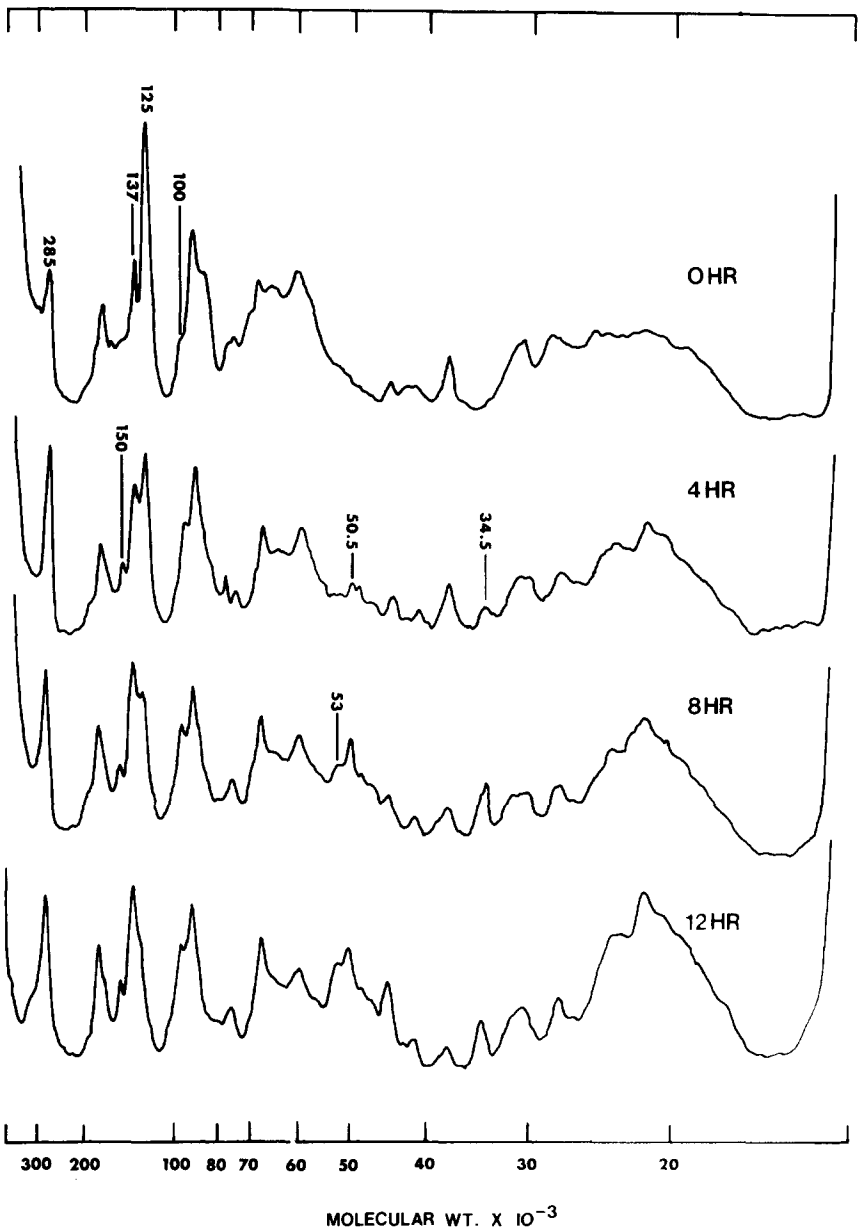

Fig. 6. Densitometric scans of the periodic acid-Schiff stained gel shown in Fig. 6. Glycoprotein components showing significant developmental changes are indicated by their approximate molecular weight $\times 10^{-3}$.

acid-Schiff reagent [27,28], 20 is a minimum estimate of the total number of plasma membrane glycoprotein components. The possible artifactual staining of non-carbohydrate-containing components has been eliminated since there was no staining of gels that were not subjected to periodic acid treatment. Furthermore, the periodic acid-Schiff staining conditions used in this study did not stain any of the standard molecular weight proteins, except ovalbumin

TABLE I

NEUTRAL AND AMINO SUGAR COMPOSITION OF *D. DISCOIDEUM* PLASMA MEMBRANES (PMI) DURING DIFFERENTIATION

Each value is the mean of 4 or 5 determinations ± S.D. Data are expressed as nmol/mg protein.

	Aggregation time (h)			
	0	4	8	12
Neutral sugars				
Fucose	38.4 ± 4.8	34.6 ± 6.2	42.5 ± 7.7	38.4 ± 6.7
Ribose	21.0 ± 19.9	29.5 ± 17.7	21.1 ± 4.6	23.5 ± 4.6
Mannose	61.8 ± 11.0	55.2 ± 5.7	62.3 ± 14.5	53.9 ± 7.8
Galactose	3.5 ± 1.8	3.6 ± 0.6	3.3 ± 1.1	3.4 ± 1.6
Amino sugars				
Glucosamine	135.1 ± 14.4			126.0 ± 9.4
Galactosamine	9.3 ± 3.8			7.3 ± 1.4

which is known to contain approximately 3.2% carbohydrate [29].

During aggregation there is a significant decrease in the amounts of gp 125, while increases are seen in gp 285, gp 150, gp 137, gp 53, gp 50.5 and gp 34.5 (Fig. 6). Several of these modifications occur during the first 4 h of differentiation. None of these modifications appear to correspond to the altered protein components (Figs. 3 and 4). The intensely staining band which migrates with the tracking dye is assumed to be glycolipid and possibly other non-proteinaceous components [21].

The analysis of plasma membrane-bound neutral and amino sugars at various stages of differentiation is given in Table I. There are no significant changes during aggregation. There are some noteworthy modifications to our previously published monosaccharide composition for purified *D. discoideum* plasma membranes [18]. The levels of glucosamine given in Table I are greater than those previously published. In the present investigation the component was quantitated on an automatic amino acid analyzer [23] as opposed to gas-liquid chromatography of the acetylated anhydrohexose derivatives [30]; the latter method in our hands gave consistently lower values, possibly because of incomplete deamination. Amino sugar analyses also revealed low levels of galactosamine, a component not previously detected by gas-liquid chromatography [18]. Also, in neutral sugar analyses, trace amounts (<2 nmol/mg protein) of a component tentatively identified as xylose on the basis of its retention time was detected.

Discussion

The cellular slime mold, *D. discoideum* undergoes a highly synchronous differentiation process when deprived of nutrients. Since the 'wild type' NC4 and axenic A3 and Ax-2 strains all exhibit essentially the same differentiation program, it is clear that the plasma membrane changes that are essential for development should be observed in all of these strains. The studies that have been performed hitherto on strains A3 and NC4 have revealed essentially no similarity in plasma membrane component modification [6,15,16] and it is

possible, therefore, that the changes described have little relevance to the developmental program.

A possible reason for the discrepancies in the results for A3 is that a different method of membrane purification was used in the two studies [6,16]. Loss of genuine plasma membrane components during the preparative procedure or contamination with internal membranes might account for the differences in the results. From the available data it is difficult to assess which of the two preparations is more representative of the native plasma membrane. More puzzling, is the lack of similarity in the studies on the developmentally regulated plasma membrane components of NC4 and A3, since the membrane preparative procedure that was employed was identical [6,15].

We have, therefore, used a plasma membrane preparative procedure which yields a well characterized, highly purified preparation [18,19], and examined the developmentally regulated modifications that occur in a third strain Ax-2 to determine if any of the previously reported developmentally regulated changes are detectable. As observed previously by other workers [6,15,16] there is considerable conservation of plasma membrane components during aggregation (Figs. 3—6). However, a number of clearly defined modifications are detectable (Figs. 4 and 6).

Some of the quantitative changes in the glycoprotein components detected in this study have been reported by previous workers. Hoffman and McMahon [16] reported an increase in the amount of a high molecular weight component (317 000 daltons) and a decrease in the amount of a component with an apparent molecular weight of 125 000. We observed a marked decrease in the amount of gp 125 and an increase in the amount of gp 285 (Figs. 5 and 6). Since there are slight inaccuracies in the determination of the molecular weights of large components, the gp 285 component is probably identical to the 317 999 dalton component of Hoffman and McMahon [16]. Furthermore the 125 000 dalton component is modified in a mutant strain that fails to aggregate [31], further implicating the developmental regulation of this glycoprotein. In addition, Geltosky and coworkers observed an increase in the amounts of a number of Con A-binding protein during aggregation [13]. They specifically eluted ^{125}I-labelled surface components from a Con A-Sepharose column with α-methyl mannoside and analyzed these components by SDS-polyacrylamide gel electrophoresis. In particular they observed a large increase in a 150 000 dalton component which probably corresponds to gp 150 (Figs. 5 and 6). They also observed an increase in the amounts of a number of low molecular weight components that appear to correspond to gp 53, gp 50.5 and gp 34.5. Neither Geltosky et al. [13] nor Hoffman and McMahon [16] reported the prominent increase in the amount of gp 137 that we observed routinely. The developmental regulation of gp 137 is therefore as yet uncorroborated.

A number of the quantitative changes in protein components observed in this study have also been reported by other workers. The appearance of p220 during aggregation probably corresponds to the developmentally regulated 220 000 dalton components described earlier by Siu et al. [6] and the decrease in the amount of p82 may correspond to the decrease in an 85 000 dalton component described by Hoffman and McMahon [16]. In addition, we see a

transient increase in the amounts of two closely migrating low molecular weight components p16 and p15.5. A similar pattern was observed for an 18 000 dalton component in NC4 plasma membranes [15] and 19 000 dalton components in the plasma membranes of A3 [6]. Finally, increased cell surface iodination of a 55 000 dalton component [5] and a 59 000 dalton component [6] has been reported as cells progress through the aggregation process. These changes may correspond to the increased amounts of p59 and p56 reported in the present study.

Despite the consistencies outlined above, a large number of other developmental changes have been reported by previous workers [6,15,16] that have not yet been confirmed by this or any of the other studies and their developmental significance remains questionable.

We have also observed that there is no qualitative or quantitative alteration in the carbohydrate moieties of the plasma membrane (Table I). Thus the aggregation-induced modification in plant lectin binding and agglutination [10—14], the alterations in the affinity of discoidin binding [14] and the quantitative variation of certain glycoproteins (Figs. 5 and 6 and ref. 16) are due to subtle rather than major changes in cell surface carbohydrate composition. Modification of a few terminal sugars or the exposure of nascent glycoproteins at the cell surface might have important developmental consequences and yet not be detected by our carbohydrate analysis. The known sensitivity of sialic acid to periodic acid-Schiff's reagent might suggest that small changes in the amounts of this residue might account for the altered patterns displayed in Figs. 5 and 6. However, this is unlikely since sialic acid is undetectable in *D. discoideum* [18].

The extensive plasma membrane changes reported here (Figs. 3—6) and elsewhere [6,15,16] may indicate that aggregation involves a number of surface components, a suggestion supported by previous genetic analysis [32—34]. Alternatively however, aggregation may be relatively simple in molecular terms, but induce extensive surface changes that are required for continued cell-cell interaction throughout the differentiation process. Considerable biochemical and genetic work will be required to determine the function of each of the developmentally regulated plasma membrane components.

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