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THE ROLE OF THE PLASMA MEMBRANE IN THE DEVELOPMENT OF *DICTYOSTELIUM DISCOIDEUM*

II. DEVELOPMENTAL AND TOPOGRAPHIC ANALYSIS OF POLYPEPTIDE AND GLYCOPROTEIN COMPOSITION

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Summary

Previous workers have shown in a variety of ways that cell contact is required for the differentiation of *Dictyostelium discoideum*. Because interactions between cells are probably mediated by molecules on their plasma membranes, we have characterized the polypeptide composition of the membrane of cells at different stages of development. At least 55 polypeptides are found in the plasma membrane of vegetative cells. The polypeptide composition of the plasma membranes changes considerably during development. Treatment of intact cells with pronase indicated that many of the altered components appear to be located on the external surface of the plasma membrane where they could participate in interactions between cells. Similar digestion of the isolated membranes destroys most of their polypeptides, indicating that the bulk of the proteins of the plasma membrane are not completely embedded in the membrane. Several polypeptides appear to change in sensitivity to pronase during development. There are several changes in glycoprotein composition which occur between log phase and aggregation phase. An almost complete change in glycoprotein species occurs between aggregation and pre-culmination. Unlike the polypeptides, the glycoproteins are very resistant to pronase treatment in intact cells. However, some are pronase sensitive in isolated membranes.

Introduction

Interactions among cells are important in development. Although the proteins and glycoproteins of the plasma membrane are widely presumed to medi-

Abbreviation: TEMED, *N,N,N',N'*-tetramethylethylenediamize.

ate at least some of these interactions, the plasma membranes of developing cells have been little studied to determine what changes occur in their protein and glycoprotein composition during development. Analysis of plasma membrane topography should also yield interesting information. Proteins directly participating in cellular adhesion or in the sensation of developmentally important environmental stimuli should be located on the external face of the plasma membrane. Determination of those proteins in the membrane which are accessible to macromolecules (such as proteolytic enzymes) in the environment can indicate proteins which could participate directly in cellular interaction.

The development of the social amoeba, *Dictyostelium discoideum*, includes a number of processes which clearly illustrate the importance of cellular interactions in development. Raper and Bonner and their collaborators [1—4] have shown that *D. discoideum* cells can distinguish homologous from heterologous species and can determine their position in the pseudoplasmodium with some precision. Sussman and his associates [5—7] have shown that cell contact is required for normal biochemical development of *D. discoideum* and for cooperation between developmental mutants. Gregg [8] has demonstrated a continuing requirement for this contact during development.

The most direct evidence for changes in the cell surface during development have come from iodination of external proteins [9] and from immunological studies of Gregg [10], Sonneborn et al. [11], and Takeuchi [12]. These studies have been elegantly expanded by Gerisch and Beug and their collaborators [13—15] who have shown that a specific adhesive site, the A site, appears on the plasma membrane during aggregation and seems to be responsible for specific cell adhesion. The chemical nature of this component has not been defined, although it may be a glycoprotein [13]. Takeuchi and Yabuno [16] have shown that pronase plus British anti-lewisite dissociates the cells of the pseudoplasmodium from one another, indicating that one or more proteins are involved in their adhesion.

Carbohydrate-containing molecules are important in the development of *D. discoideum*. Gillette and Filosa [17] showed that concanavalin A inhibited the aggregation of strain NC4 and that it prematurely induced a cyclic AMP phosphodiesterase. A 4-fold increase of activity was produced within 30 min of treatment. The induction was prevented by α-methylmannoside, indicating that the lectin produced its effect by interacting with a carbohydrate receptor, and by actinomycin D, suggesting that the transcription of a gene was required for the increase of activity. Weeks and Weeks [18] showed concanavalin A had similar effects on strain Ax-2 and were able to dissociate the inhibition of aggregation from the premature induction of cyclic AMP phosphodiesterase.

A theory for the mechanism by which cells determine their position in the pseudoplasmodium has recently been proposed. It suggests that this information results from the interaction of complementary molecules on apposing cell surfaces that regulates the intracellular concentration of a morphogen such as cyclic AMP [19]. Some of the predictions of this theory have recently been confirmed [20—23] increasing the potential interest in investigations of the developmental regulation of the components of plasma membrane.

Materials and Methods

Buffers and chemicals. Tris/magnesium buffer is 20 mM Tris · HCl (pH 8.0) and 5 mM $MgCl_2$. Denaturation buffer, modified from Fairbanks et al. [24] is 4% sodium dodecyl sulfate, 4% mercaptoethanol, 20 mM Tris/acetate (pH 8.0), 40% sucrose and 10 μg/ml pyronin Y. Homogenization buffer, 0.39 M sucrose, 10 mM Tris · HCl, 10 mM $MgCl_2$, 0.1 mM EDTA, pH 7.9, was saturated with phenylmethyl sulfonyl fluoride before use (to inhibit proteases) by shaking a few crystals of phenylmethyl sulfonyl fluoride in it. Pronase-CB and British anti-lewisite were obtained from CalBiochem. Phenylmethyl sulfonyl fluoride was the product of Schwartz/Mann and Renografin-76 was a product of Squibb, Inc. Streptomycin was produced by Eli Lilly and Co. Acrylamide and bisacrylamide, the products of Eastman Kodak, were recrystallized from chloroform and acetone, respectively, before use.

Growth and development of cells. D. discoideum, strain A-3, were grown in HL-5 medium [25] at 22°C and were harvested by centrifugation when they reached a concentration of $5 \cdot 10^6$—$1 \cdot 10^7$ cells/ml. Cells in early aggregation phase were produced by shaking cells ($1 \cdot 10^7$ per ml) 12 h in aggregation buffer [26]. Cells in pre-culmination stage were prepared by plating on Whatman filter paper as described by Newell et al. [27] with some modification. Their lower pad solution was diluted by 50% with water and 100 μg/ml of streptomycin was added. After 18 h the cells were scraped from the filters with a spatula and residual cells washed with cold diluted lower pad solution.

Preparation of membranes. Plasma membranes were prepared as described in the paper preceding [28].

Treatment with pronase. Cells ($5 \cdot 10^8$ per ml) equivalent to about 1 mg/ml of plasma membrane protein were shaken in 0.15 M NaCl containing 1 mg/ml of pronase and 3 mM British anti-lewisite for 30 min at 22°C. Two volumes of homogenization buffer (4°C) were added and the cells were centrifuged at $2000 \times g$ for 1 min. They were washed three more times with homogenization buffer and membranes were prepared from them. Freshly prepared membrane (15 mg protein/ml) were also treated with pronase as above and washed and centrifuged three times in Tris/magnesium buffer plus phenylmethyl sulfonyl fluoride at $48\,200 \times g$ for 10 min.

Preparation and use of sodium dodecyl sulfate-polyacrylamide exponential gradient slab gels. Glass plates were thoroughly cleaned with a series of washes with concentrated KOH, chromic acid, and 1% (w/v) sodium dodecyl sulfate and rinsed with 100% ethanol. When dry, 0.14 cm thick plastic spacer sticks were clamped between the plates with a little silicone grease at the junctions of the sticks to form a well 13.4 cm across. Layers of silicone grease and 2% agar were put on the outer edge of the spacers in order to insulate the gel against transverse electrical current and to stop leakage of the unpolymerized gel mix.

An 8—15% acrylamide exponential gradient gel was poured by the method of Van Blerkom and Manes [29] using a peristaltic pump to control the flow rate. 24 ml of 8% acrylamide was put in the rear chamber of the gradient maker and 10.56 ml of 15% acrylamide in the front chamber. When the rear chamber emptied, the pump was turned off. The constitution of the gel mix, reservoir buffers, and sample buffer was that of Laemmli [30] with the following excep-

tions. The final concentrations of TEMED and $(NH_4)_2S_2O_8$ in the separating gel were 0.025% (v/v) and 0.03% (w/v), respectively. In the stacking gel of 4.5% acrylamide, TEMED was increased to 0.1% (v/v). The Tris buffer used in the stacking gel and the sample buffer was adjusted to pH 5.7 instead of 6.8. The tracking dye used was pyronin Y.

Following pouring, the gel was carefully overlaid, with a Hamilton syringe, with Tris buffer of the same final concentration and pH as that in the separating gel, i.e. 0.375 M Tris · HCl (pH 8.8). The gel polymerized in about 35 min. After 75 min, the unpolymerized material was removed and a stacking gel 1—1.5 cm in length was poured around a comb that formed 17 wells. After 30 min, the comb was removed and the gel was ready to use. Gels that had been allowed to polymerize overnight showed the same pattern of bands, but tended to form ridges in the gel and showed extra skewed bands, which were artifacts, although the gels were stored at high humidity.

Membrane protein samples of 50 μg protein in a volume of 25 μl were loaded into the desired wells. Electrophoresis was at 10 mA for 1.5 h until the tracking dye was about 1 cm into the separating gel, and then at 20 mA for an additional 3.25 h. These gels were stained by the method of Weber and Osborn [31]. The results shown are gels representative of the results of a total of at least three experiments. The proteins thyroglobulin (335 000 daltons), myosin (220 000 daltons), β-galactosidase (135 000 daltons), phosphorylase *b* (92 500 daltons), human serum albumin (68 000 daltons), pig γ-globulin heavy and light chains (50 000 and 23 000 daltons, respectively), cytochrome *c* (11 800 daltons), and α-bungarotoxin (7904 daltons) were used to generate a calibration curve for molecular weights.

200 μg of membrane protein in 25—75 μl of sample buffer were routinely run in each lane for glycoproteins. Gels were stained for carbohydrate by the periodic acid-Schiff method as described by Glossmann and Neville [32] except that the gels were destained by simple diffusion. Several observations indicated the specificity of this system for glycoproteins. Two glycoprotein standards, glucose oxidase and avidin, stained intensely in this system while protein standards, cytochrome *c* and phosphorylase *b* did not stain (Fig. 5, lane 1, arrows mark the expected positions of phosphorylase *b* and cytochrome *c*). Furthermore, there was no resemblance between the pattern of periodic acid-Schiff staining and that of Coomassie blue protein staining for either plasma membrane or unfractionated cells (compare Figs. 2 and 3 with Figs. 4 and 5). However, as found by Glossmann and Neville [32] if a gel was allowed to remain for several weeks in the destaining solution without a change of solution, an artifactual pattern of staining appeared that was indistinguishable from the Coomassie blue staining pattern. In order to test whether the putative glycoproteins did in fact contain protein we incubated plasma membranes with pronase in the electrophoresis dissociation buffer (which contains sodium dodecyl sulfate) for 6 h at 22°C.

The results shown are intact representative gels from a total of at least three experiments.

Results

Changes in polypeptides during development

The plasma membrane of log phase amoebae contains about 33 polypeptides (Fig. 1a) and changes in composition during development (Figs. 1b and c) when examined by the method of Fairbanks et al. [24]. When exponential gradient gels are used increased resolution is obtained. 55 bands can easily be resolved in Fig. 2 and visual examination of the gels indicates that there are at least 16 more bands. The spectrum of polypeptides in the plasma membrane of log phase amoebae (Fig. 2, lane 8) is substantially different from that of whole cells (Fig. 2, lane 7). The major polypeptide band in whole cells (to the left of D in Fig. 2) appears to be actin [33]. A component of the plasma membrane (band X) comigrates with the major polypeptide of total cells and with the actin component of purified actomysin from *Physarum polycephalum.* This band may in fact be actin since actin is a component of partially purified membranes [34] and since the actin fibers are associated with the membrane in *D. discoideum* amoebae [35]. On the basis of its comigration with pure discoidin and its adsorption to agarose, which is reversed by D-fucose, a hapten for discoidin [36], polypeptide S may be discoidin [37].

We can only resolve five differences at most between the polypeptide composition of log phase and aggregation phase cells with Fairbanks gels (Fig. 1a). A greater number of differences are apparent using exponential gradient gels (Fig. 2, lane 9). Eight polypeptides (A—E, G, J, K) declined in relative amount between log and aggregation phases. Several polypeptides increase or appear in aggregation phase (M, N, a). Two other bands (Q, R) increase in aggregation phase but are not as apparent on Fig. 2 as on Fig. 3.

When cells passed from aggregation phase to pre-culmination, additional changes in the plasma membrane occurred (Figs. 1 and 2). These were especially clear on the exponential gradient gels. Several bands continued to decrease (A, B, G). In addition bands L—P, S—U, and Z decreased. Although not apparent in the photographs a very large polypeptide (M_r = 313 000) decreases from aggregation to pre-culmination phase. Five polypeptides (V—Y, c) appear or increase in pre-culmination phase. The molecular weights of the polypeptides which change in development are presented in Table I.

Sensitivity of the polypeptides to pronase

We have digested cells and membranes with pronase to resolve the elementary topography of the plasma membrane for several reasons. This method does not require exposure of a specific amino acid as iodination does. It requires only that some portion of the polypeptide, more than 10% from either end, be exposed to the action of enzymes. Pronase treatment has been shown to be specific and reliable for identifying external membrane polypeptides [38,39]. Several facts support its reliability in our system. The pronase treatment does not decrease cellular viability (ref. 16 and our unpublished results); treatment of intact cells does not alter the respiration rate of the treated cells [16]; and it does not reduce the activity of the lactate dehydrogenase that they contain (Table II). So it is unlikely that the treatment damages proteins inside the cell.

Exponential gradient gel electrophoresis (Fig. 3) showed that six polypep-

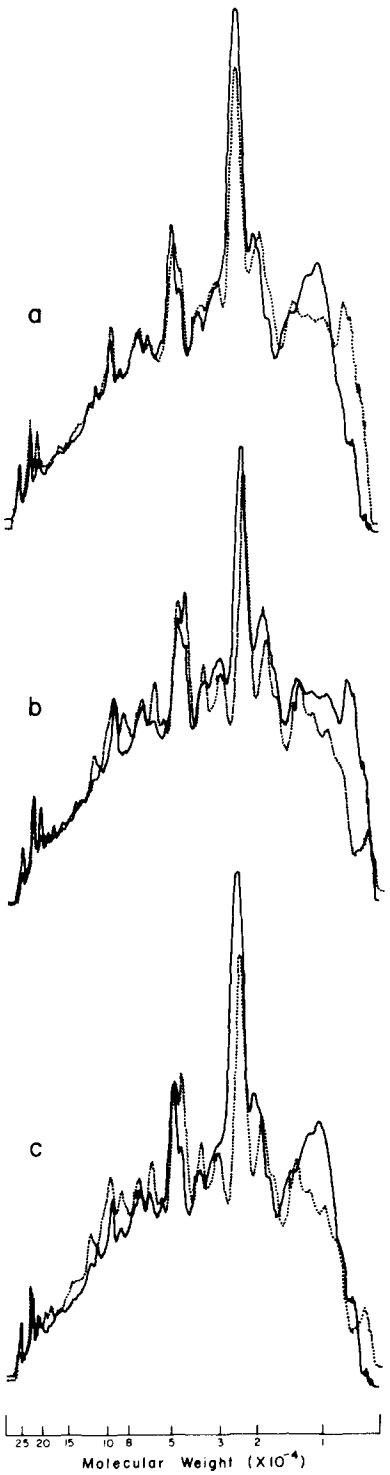

Fig. 1. Alterations of plasma membrane polypeptides during development. Densitometric scans of gels prepared by the method of Fairbanks et al. [24] and stained with Coomassie brilliant blue are superimposed to illustrate changes in polypeptide composition of the plasma membrane. (a) Log phase (solid line) vs. aggregation phase (dotted line); (b) aggregation phase (solid line) vs. pre-culmination phase (dotted line); (c) log phase (solid line) vs. pre-culmination phase (dotted line).

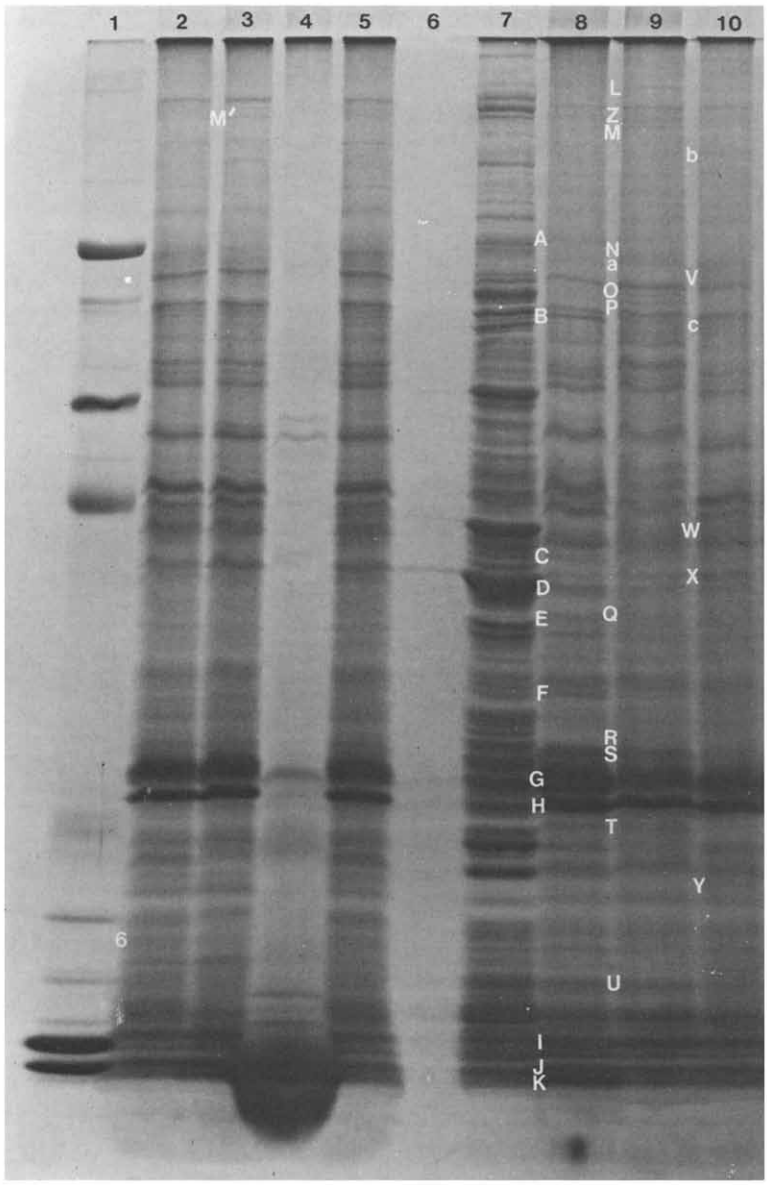

Fig. 2. Exponential gradient gel electrophoresis of polypeptides. Lanes from left to right are: (1) standard proteins as described in Materials and Methods; (2) plasma membranes from pre-culmination cells; (3) plasma membranes from pronase-treated pre-culmination cells; (4) pronase-treated plasma membranes from pre-culmination cells; (5) as in 2; (6) blank; (7) total protein from log phase amoebae; (8) plasma membranes from log phase amoebae; (9) plasma membranes aggregation phase cells; (10) plasma membranes from pre-culmination cells. Polypeptides which change in amount during development are identified by letters. Those whose sensitivity to pronase appears to change during development are numbered. Band F appeared to decrease continuously during development on this gel; however, this result was not repeatable on other gels.

tides of the amoebal plasma membrane which change during development (B, C, E, L, P, Z) are sensitive to pronase. Band 1 which is not developmentally regulated is also pronase sensitive in the intact cell. Bands 2 and T were variably

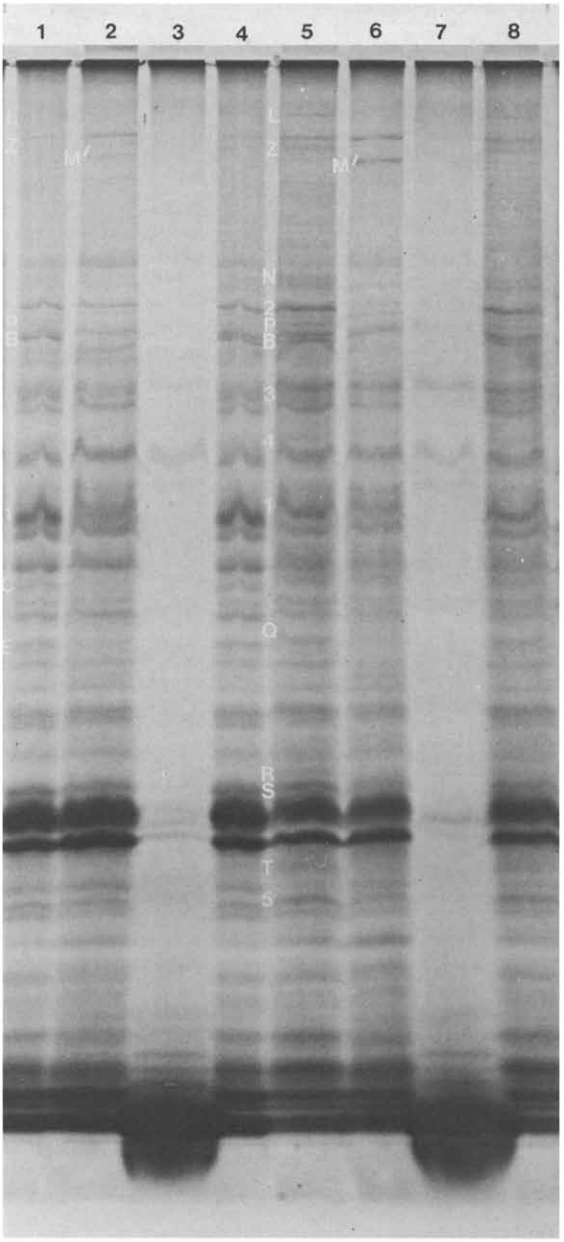

Fig. 3. Exponential gradient gel electrophoresis of polypeptides from log and aggregation phase plasma membranes. Lanes are from left to right: (1) log phase plasma membranes; (2) plasma membranes from pronase-treated log phase cells; (3) pronase-treated plasma membranes from log phase cells; (4) as in 1; (5) plasma membranes from aggregation phase cells; (6) plasma membranes from pronase-treated aggregation phase cells; (7) pronase-treated plasma membranes from aggregation phase cells; (8) as in 5.

(two of three preparations) pronase sensitive while band S (which co-migrates with discoidin [37]) was sensitive in one of three preparations. A new polypeptide, M′, of 211 000 molecular weight is present on pronase-treated mem-

TABLE I

MOLECULAR WEIGHTS OF POLYPEPTIDES WHICH CHANGE DURING DEVELOPMENT AND/OR ARE SENSITIVE TO PRONASE IN INTACT CELLS

Symbol	Molecular weight ($\times 10^{-3}$)	Sensitivity to pronase					
		Log	Aggregation		Pre-culmination		
Bands which change during development							
A *	122	—	—	↓	NP		
B	85.1	+	+	↓	—		
C	45.0	+	NP		NP		
D	41.0	—	—	↓	—		
E	38.6	+	NP		NP		
G	24.4	—	—	↓	—	↓	
H	22.9	—	—	↓	—	↓	
I	11.9	—	—		—		
J	11.2	—	—		—		
K	10.9	—	—	↓	—		
L	265	+	+		NP		
M	211	NP	—		NP		
N	114	NP	+		NP		
O	92.7	—	—		NP		
P	89.6	+	+		NP		
Q	39.2	NP	+		—		
R	27.8	VP+	+		—		
S	26.8	+/—	+		—	↓	
T	21.7	+/—	+		NP		
U	14.0	—	—		—	↓	
V *	104	NP	NP		—		
W	48.6	NP	NP		—		
X	43.1	—	—		—	↑	
Y	18.9	NP	NP		—		
Z	222	+	+		NP		
a	111	NP	—		—		
b	195	NP	NP		—		
c	84.1	NP	NP		—		
Bands whose sensitivity to pronase changes during development							
1	53.8	+	+/—		—		
2	96.6	+/—	+		—		
3	73.8	—	+/—		—		
4	66.1	—	+		—		
5	19.9	—	+		—		
6	15.4	—	—		+		

* Band is a doublet on some gels.
+, sensitive to pronase.
—, not sensitive to pronase.
+/—, variably sensitive to pronase.
NP, not present in untreated membranes.
↑, increase in apparent amount of polypeptide.
↓, decrease in apparent amount of polypeptide.
VP, variably present.

branes. It may represent a fragment of a larger polypeptide or a new polypeptide which attaches to the plasma membrane as a result of the treatment with pronase. Finally band A increases in intensity after treatment with pronase.

When membranes which have been isolated from log phase cells are treated

TABLE II

ACTIVITY OF LACTATE DEHYDROGENASE IN PRONASE-TREATED AND CONTROL CELLS

Intact cells were treated with pronase as described in Materials and Methods, or were untreated. They were harvested, washed, and sonicated, and lactate dehydrogenase activity of the extract was assayed [50].

Treatment	Activity (μmol NADH/min)	
	per 10^8 cells	per mg protein
Untreated	70	6.7
+ pronase	70	7.0

with pronase very few high molecular weight polypeptides remain. Two of these which have molecular weights of 75 800 and 62 100 co-migrate and are nearly as intense as the corresponding bands in the untreated plasma membrane. Two bands (molecular weights of 122 400 and 89 000) are present but in considerably diminished amount. A component of molecular weight 57 900 is also present. Only the polypeptide of molecular weight 62 100 is likely to be an intramembraneous protein, since the other pronase-resistant components co-migrate with glycoproteins which are resistant to pronase (see below). With the exception of six low molecular weight fragments all of the residual protein migrates more rapidly than cytochrome *c*.

When intact aggregation phase cells were treated with pronase, 14 polypeptides were destroyed. These are marked in Fig. 3 and identified in Table I. Two of these bands, 1 and 3, were variably sensitive. Three of the sensitive polypeptides (N, Q, R) have increased or appeared by aggregation phase. Some proteins, band 5 is particularly evident, were found in other phases of development but were pronase sensitive only in aggregation phase. Ten bands (1—6, B, Q—S) with similar behavior are described in Table I. Bands M′ and A increase in amount again after the treatment with pronase. When isolated membranes were treated with pronase, the pattern of remaining polypeptides resembled that of log phase cells.

Protease treatment of pre-culmination phase cells reproducibly destroys band 6. A band with identical mobility is also present in both log and aggregation phases but is not pronase sensitive. One other protein (molecular weight 234 000) is variably sensitive. The band M′ increases after treatment. Few polypeptides are evident where the isolated membranes are treated with pronase. There are noticeable differences when compared with similarly treated membranes from other stages. Most notably a band whose molecular weight is 64 300 remains after the treatment with pronase.

Changes in glycoproteins during development

Fig. 4 (lane 2) shows that the plasma membrane fraction of log phase amoebae contains at least fifteen glycoproteins. Some of these glycoproteins stain faintly. The pattern of glycoproteins is very reproducible (compare lanes 9 and 10 in Fig. 4). There is only a single difference between them, the very faint band labeled V. It is obvious that the glycoproteins contain only a small

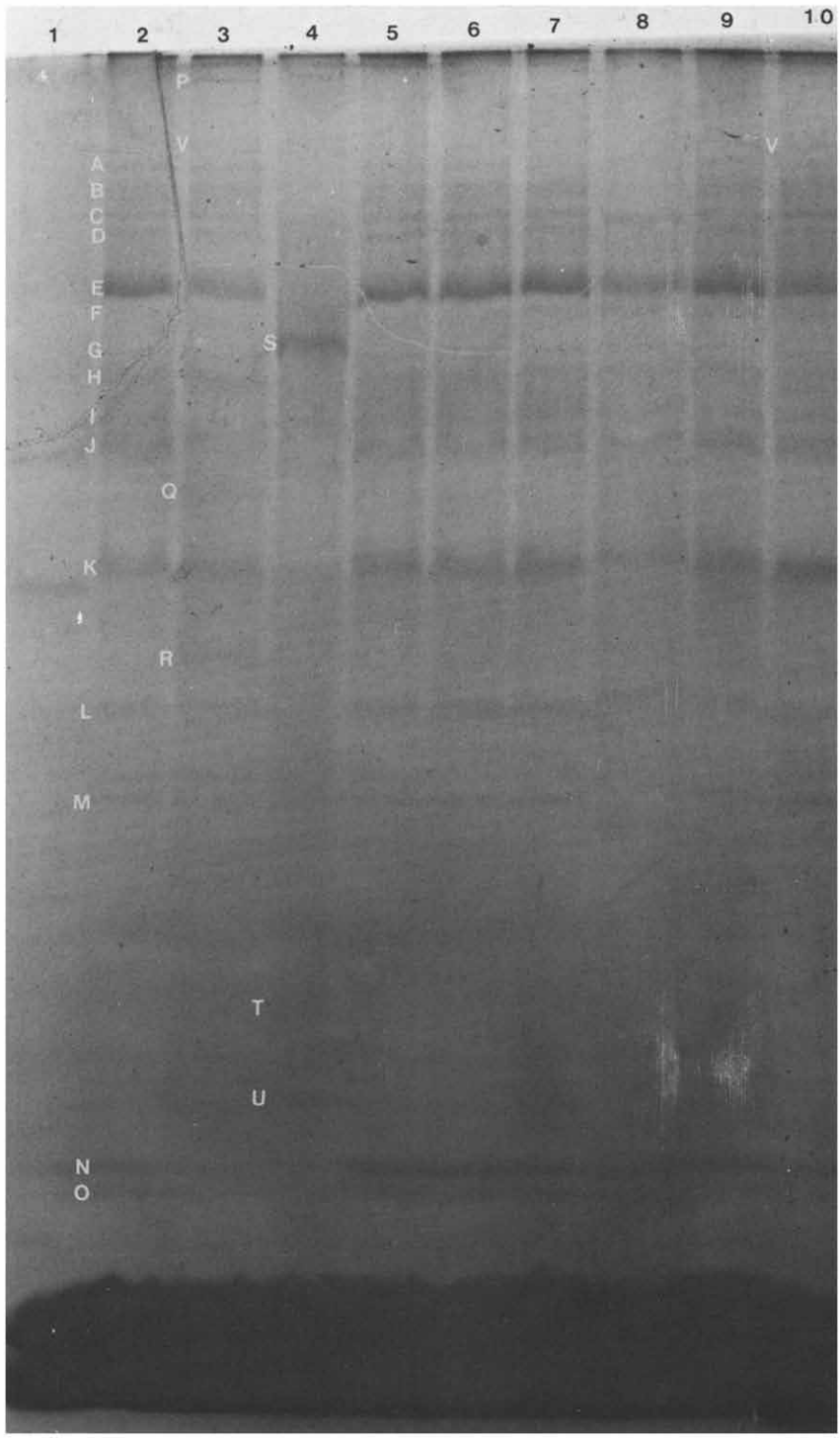

Fig. 4. Exponential gradient gel electrophoresis of glycoproteins. Lanes from left to right are: (1) total protein from log phase amoebae; (2) plasma membranes from log phase amoebae; (3) plasma membranes from aggregation phase cells; (4) plasma membranes from preculmination phase cells; (5) as in 2; (6) as in 2; (7) plasma membranes from pronase-treated log phase amoebae; (8) pronase-treated plasma membranes from log phase amoebae; (9) as in 2; (10) plasma membranes from log phase amoebae (independent preparation).

fraction of the total material which stains with the periodic acid-Schiff reagent. The majority of material runs close to the dye front and may by glycolipids although we have not characterized it. In addition some material which stains with the periodic acid-Schiff reagent does not enter the gel. All of the stained bands including the material that does not enter the gel but excluding the possible glycolipid band are destroyed by pronase treatment of membranes dissociated by sodium dodecyl sulfate. When plasma membranes are purified they are significantly enriched for carbohydrate-containing molecules. An equal amount of protein from entire log phase amoebae which were dissociated and electrophoresed showed only one stained band (Fig. 4, lane 1). The other apparent band is a photographic artifact caused by the rip in the gel. Therefore, the glycoprotein bands are unlikely to result from adsorbed cytoplasmic glycoproteins. This conclusion is also supported by our preliminary experiments which indicate that washing the plasma membranes with 1 mM EDTA or deionized water does not remove the glycoproteins from the membranes.

The plasma membranes of aggregation phase cells exhibit several differences.

TABLE III

MOLECULAR WEIGHTS OF GLYCOPROTEINS

Symbol	Apparent molecular weight ($\times 10^{-3}$)	Presence during development			
		Log	Aggregation	Pre-culmination	Possible corresponding protein
A	214	+P	+P	—	
B	195	+P	+P	—	
C	178	+	+	—	
D	155	+P	+P	—	
E	124	+	↓	—	A
F	113	+	+	—	
G	96.0	+	+	—	
H	86.9	+P	+P	—	
I	79.5	+	—	—	
J	75.1	+P	+P	—	
K	58.1	+	+	↓	
L	42.2	+	+	—	
M	34.5	+P	+P	+P	
N	15.6	+	↓	↓	
O	14.6	+	+	↓	
P	317	+/—P	+P	—	
Q	68.1	—	+	—	
R	47.8	—	+	—	
S	101	—	—	+	
T	22.4	—	—	+	
U	18.0	—	—	+P	Y
V	237	+/—	+P	—	
W	79.1	—	—	+	
X	72.3	—	—	+	
Y	63.8	—	+/—	—	

+, present.

↓, present, but in a decreased amount than in the previous stage.

—, not present.

P, sensitive to pronase in purified membranes.

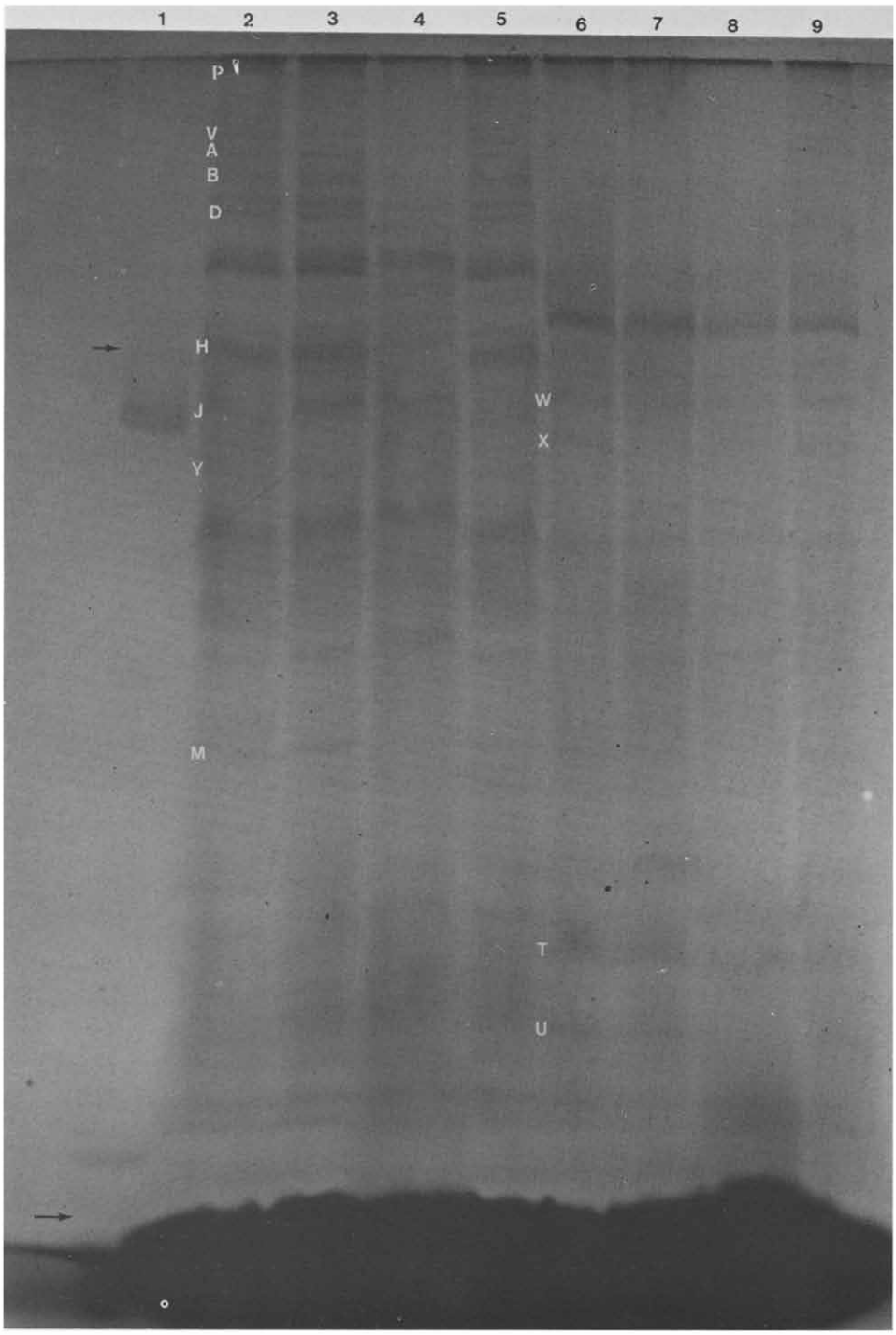

Fig. 5. Exponential gradient gel electrophoresis of glycoproteins from aggregation and pre-culmination stage plasma membranes. Lanes from left to right are: (1) phosphorylase *b*, glucose oxidase, avidin, cytochrome *c* (2.5 μg each), arrows mark the position of phosphorylase *b* and cytochrome *c*; (2) plasma membranes from aggregation phase cells; (3) plasma membranes from pronase-treated aggregation phase cells; (4) pronase-treated plasma membranes from aggregation phase cells; (5) as in 2; (6) plasma membranes from pre-culmination phase cells; (7) plasma membranes from pronase-treated pre-culmination phase cells; (8) pronase-treated plasma membranes from pre-culmination cells; (9) as in 6.

Band I disappears. Bands E and N decrease in intensity although they are still present. A new glycoprotein (P) and two faint bands (Q, R) are evident in addition to those remaining from the log phase cells.

The plasma membranes of pre-culmination phase cells are strikingly different from those of log and aggregation phase cells (Fig. 4, lane 5). With the exception of bands K, M, N and O, all of the old glycoprotein bands disappear. Of those that have remained all except band M seem to have decreased in amount. Three new glycoproteins (S—U) appear reproducibly.

The migration of some glycoproteins on sodium dodecyl sulfate-polyacrylamide gels is not strictly a function of their molecular weights [32]. Therefore, we have presented apparent molecular weights in Table III for the bands described above.

Sensitivity to pronase

No glycoprotein bands were unambiguously sensitive to pronase in entire cells (Figs. 4 and 5). This contrasts strikingly with the sensitivity to pronase of many of the plasma membrane polypeptides which do not contain detectable carbohydrates.

Treatment of isolated log phase plasma membranes with pronase destroyed bands A, B, D, and M. Bands H and J were present, but reduced in amount. There may be some limited destruction of other glycoproteins, however, since they are approximately equal in intensity to untreated membranes after treatment. This was contrary to our expectations since plasma membranes from twice as many cells are used in the lanes containing samples which were treated with pronase in vitro since the treatment with pronase releases 50% of the protein from the isolated membranes.

When plasma membranes from aggregation phase cells were treated with pronase (see lane 4, Fig. 5), bands A, B, D, and M (which were conserved from log phase) continued to be degraded. Bands P, V, and H are also degraded by pronase. Band J is partially sensitive as in log phase. An apparent fragment whose mobility is greater than band D appears after the treatment.

Band U from pre-culmination phase plasma membranes is sensitive to pronase in isolated membranes. The general resistance of the glycoproteins to pronase in all phases of development is very different from the almost total sensitivity of the polypeptides.

Discussion

The polypeptide and glycoprotein composition of the plasma membrane changes considerably during the first 18 h of development. Before considering any of our results, we wish to consider some trivial explanations for them. We might be observing a changing pattern of adsorbed cytoplasmic proteins but, for two reasons, this does not appear likely. Many of the changes occur in polypeptides which are destroyed by treating the intact cell with pronase and consequently seem to be on the outside of the cell. Also, adsorbed macromolecules would probably be in the class of molecules, including extrinsic polypeptides and glycoproteins [40] which can be eluted from the membrane by non-denaturing solvents. We have eluted purified membranes with EDTA at

neutral and basic pH values and with deionized water. A number of components which change in amount in development which are not eluted (including, for example, the 122 000 and 313 000 polypeptides of log phase cells) are intrinsic polypeptides by this criterion (unpublished results). However, some minor molecules may be components of the small amount of contaminating mitochondrial inner membrane. We will publish studies elsewhere which indicate that many of the changes (including decreases in amount of a component) are coupled to development and are, in general, unlikely to simply result from some trivial cause such as starvation.

Since any vicinal glycol can be oxidized by periodate [41], we should be able to stain all glycoproteins that have a glycol grouping anywhere in their carbohydrate structure. The glycoprotein standards, avidin and glucose oxidase, stain intensely in this system but the proteins cytochrome *c* and phosphorylase *b* do not. Some indication of the sensitivity of our method is given by the staining of the standard glycoproteins. In the case of avidin (the band with greater mobility in Fig. 5, lane 1) assuming a carbohydrate content of 9% [42], this band contains 0.225 μg carbohydrate. Our lower limit of resolution could be much less. This amount of carbohydrate would amount to about 0.1% of the mass of protein in the membranes which were electrophoresed.

The accuracy with which external polypeptides can be identified on the basis of their sensitivity to pronase in the intact cell deserves consideration. This assumption is supported by the absence of detectable deleterious effects of pronase on the intact cell and by the studies of vertebrate cells which indicate that this method accurately identified external polypeptides [38,43,44]. The possibility remains, however, that pronase could modify the membrane in a way which causes the release of extrinsic polypeptides bound to the cytoplasmic face of the membrane or stimulate the adsorption of new polypeptides from the cytoplasm. Band M′ could be such a polypeptide. Alternatively it may represent a fragment cleaved from the material which does not enter the gel.

By aggregation phase, the pattern of the plasma membrane polypeptides (particularly those on the outside) had changed considerably. Some alteration may occur in the environment of some of the polypeptides (bands 3—5) since they are sensitive to pronase only at this stage. Alternatively, these bands may be cryptic substitutions of new polypeptides with identical mobilities for polypeptides previously present which were insensitive to pronase. It is interesting that band S (which co-migrates with purified discoidin) is sensitive to pronase in aggregation.

Cells in the pseudoplasmodium and pre-culminate are engaging in pattern formation and the final stages of morphogenesis. The effects of pronase treatment indicate that few polypeptides are sensitive to pronase at this stage. Band 6 is the only polypeptide which is clearly sensitive to pronase. Nine polypeptides (1—5 and B, Q—S) seem to have lost their sensitivity to pronase. Again it is not clear whether these bands are new polypeptides which have replaced an insensitive polypeptide and whose presence is revealed by the pronase treatment or whether their environment or location on the membrane have been modified. Fingerprinting of these bands should distinguish between these possibilities. The presumptive actin band (X) increases in amount at this time. Tuchman et al. [33] have shown that actin is the major protein synthesized

during aggregation phase. These results suggest that more actin is also attached to the plasma membrane. There is no clear correspondence between the external polypeptides we have identified and those identified by Smart and Hynes [9].

The apparent change in the sensitivity of many polypeptides to pronase during development was unexpected. The observed changes might be the result of cryptic substitution for one polypeptide of another with a different sensitivity to pronase but because of the number of such events and because, in the cases of five polypeptides, at least two cryptic substituttions per polypeptide would be necessary, it appears unlikely that all of the observed changes are caused by cryptic substitutions. Nevertheless, the result must be confirmed using a method which is sensitive to the primary structure of the polypeptides.

No such changes in the topography of the plasma membrane during development have been reported previously, although two cases in which similar topographical changes occur during the cell cycle have been investigated. Varga et al. [45] have shown that the accessibility of the melanocyte-stimulating hormone receptor apparently changes during the cell cycle. Gahmberg and Hakomori [46] have shown that the accessibility of several glycolipids and a glycoprotein to galactose oxidase changes during the cell cycle in hamster NIL cell lines.

Every glycoprotein except one changes during the first 18 h of development. Some of these changes may represent modifications of previously present glycoproteins. For example, their mobility could change as a result of removal or addition of carbohydrate. Such changes, however, would be expected to affect the biological activity of the molecule. Three glycoproteins appear at aggregation stage. At pre-culmination stage, with one exception, an entirely new spectrum of glycoproteins is seen than were present at aggregation stage. The developmental changes in plasma membrane glycoprotein composition are much more striking than those in protein composition.

No glycoproteins were clearly sensitive to pronase in intact cells. Only about one-half of the glycoproteins were sensitive in purified membranes. In these respects, glycoproteins are very different from polypeptides. The striking resistance of *D. discoideum* glycoproteins to pronase is in marked contrast to the sensitivity of glycoproteins in other systems, such as the erythrocyte [49]. The sugar moieties of glycoproteins in other cells are generally believed to be located at the external face of the plasma membrane[47,48]. We believe that at least some of the glycoproteins of *D. discoideum* are located on the external face of the plasma membrane, but that the carbohydrate part of the molecule protects the polypeptide part from degradation by pronase. The sensitivity of the putative glycoproteins incubated in sodium dodecyl sulfate plus 1 mg/ml pronase described above agrees with this postulate. Under this condition, pronase destroyed the glycoproteins (Hoffman, S., unpublished results). If the assumption is made that glycoproteins are protected from pronase degradation at the cell surface by their carbohydrate moieties, then some or all of the glycoproteins that are pronase sensitive in purified membranes must traverse the membrane. Our finding that some glycoproteins are unaltered by pronase treatment of the cell suggests that one of the new glycoproteins which appear aggregation phase could correspond to contact sites A, as described by Beug et al. [13].

Two glycoproteins may correspond to polypeptides on the basis of their identical apparent molecular weights, their sensitivity to pronase, and their changes during development. These provisional correspondences are presented in Table III. The glycoproteins H, J, and K may correspond to the pronase-resistant polypeptides of molecular weights 89 000, 75 800, and 57 900 observed in Fig. 3.

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