THE ROLE OF THE PLASMA MEMBRANE IN THE DEVELOPMENT OF THE CELLULAR SLIME MOLD, *DICTYOSTELIUM DISCOIDEUM*

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ABSTRACT

In order to evaluate the role of the plasma membrane in the development of the cellular slime mold, Dictyostelium discoideum, plasma membrane composition and topography were studied in wild type (A3) cells and cells inhibited in development including cells of an aggregation minus mutant, HM 2, with abnormal cellular interactions. One molecule of particular interest, glycoprotein E, was purified and transferred from A3 cells to HM 2 cells and vice versa in order to study the feasibility of experimentally altering plasma membrane composition.

The plasma membrane polypeptide composition and topography of vegetative A3 and HM 2 cells are very similar. However, more sensitive probes can detect several differences between A3 and HM 2. Differences were found between vegetative A3 and HM 2 plasma membranes in antigenic macromolecules, Concanavalin A (Con A) receptors, periodic acid Schiff (PAS) positive glycoproteins, and a glycolipid. Glycoprotein E was aberrant in HM 2 both antigenically and as a Con A receptor. Three PAS positive glycoproteins, including glycoprotein E, were more sensitive to protease treatment of HM 2 than A3 plasma membranes.

Purified glycoprotein E is homogeneous on SDS gels, forms a broad band during isoelectric focusing, and contains a high level of polar amino acids. Glycoprotein E in lipid vesicles is differentially incorporated by A3 and HM 2 cells. The site of glycoprotein E incorporation is the plasma membrane of target cells as demonstrated by indirect immunofluorescence. This study also indicated that this antigenic form

of glycoprotein E is not uniformly exposed on the surface of all A3 cells.

The plasma membrane macromolecular composition and topography of A3 cells changes during development. Forty percent of the polypeptides and ninety-five percent of the glycoprotein species change in amount present during development to preculmination. About 15 cell surface polypeptides were detected at various stages of development, several of which apparently change their topographical location during development.

When development is inhibited by the use of HM 2 cells or cycloheximide treatment of A3 cells, most developmental changes in plasma membrane composition and in polypetide topography were also blocked. In addition, unexpected changes in plasma membrane composition and topography occurred. For example, in inhibited cells, several glycoproteins were more sensitive to pronase treatment of isolated membranes than in A3 cells. Cycloheximide treatment caused the unexpected disappearance of several cell surface polypeptides from the plasma membrane. Although cycloheximide treatment was most effective in blocking developmental changes in plasma membrane composition, the HM 2 mutation had a greater effect on the disruption of plasma membrane topography.

Therefore, these studies indicate that several changes in plasma membrane composition and topography appear to be an integral part of development. Furthermore, in the HM 2 mutant, defects in the plasma membrane even before the initiation of development are

correlated with abnormal cellular interactions. Lastly, the feasibility of studying the role of the plasma membrane in development by experimentally altering plasma membrane composition has been demonstrated.

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GENERAL INTRODUCTION

I. Development of Dictyostelium discoideum

The cellular slime mold, D. discoideum, is an excellent model system for studying the role of cell contact in eukaryotic cytodifferentiation and pattern formation. Large quantities of cells can be grown either on bacteria or axenicly, and then caused to differentiate synchronously by starvation. Mutant clones, blocked at a variety of developmental stages, can easily be scored by visual examinations of plaques formed on a bacterial lawn by mutagenized or untreated cells. Therefore, the development of D. discoideum is very amenable to biochemical analysis.

Three developmental pathways are available to D. discoideum cells when environmental conditions become unfavorable for growth. The most widely studied pathway, the only one studied in this thesis, is the sorocarp pathway. This pathway is promoted by light, phosphate buffer, an air-water interface, and relatively low temperature ($< 20^{o}C$). Under these conditions from 10^1 to 10^5 cells, depending on local cell density, come together and form a strongly cohesive aggregate. This process takes 8 to 12 hours. Over the next 4 hours the aggregate extends itself into a slug-like pseudoplasmodium or grex. At this stage the prestalk and prespore cells are already localized respectively to the anterior and posterior regions of the pseudoplasmodium (1). However, the developmental fate of these cells is still plastic. If allowed to continue development, the pseudoplasmodium will right itself and the prestalk region will push down through the prespore mass until it comes in contact with

the substratum. The developing ball of spores then rises up the outside of the stalk until at the end of a total of 24 to 30 hours of development, the final sorocarp consists of a ball of individually enclosed spores on top of a stalk consisting of dead, vacuolate cells.

If a pseudoplasmodium is cut into anterior and posterior segments, the posterior segment will develop normally while the anterior segment will be partially deficient in spore cells (2). However, if the anterior segment is put under conditions that maintain the pseudoplasmodium stage of development for 18 hours, when culmination occurs the resulting sorocarp is normally proportioned (2).

In submerged cultures, the sorocarp pathway can proceed to the aggregation stage (3). Recently, it has been reported that stalk and spore cytodifferentiation and pattern formation similar to that seen in the pseudoplasmodium can occur in this system (4, 5).

The other pathways available to D. discoideum amoebae under unfavorable conditions are the microcyst pathway in which individual amoebae encyst themselves and the macrocyst pathway, a sexual cycle that requires cell contact.

II. The Role of Cell Surface Molecules in Development

Several types of interactions that must involve cell surface molecules occur during the development of D. discoideum. When an aggregate forms, the cells involved become highly cohesive.

This activity has been assigned to the immunologically defined contact site A (6). The specificity of these contacts is indicated by the fact that different species of slime mold that are coaggregated either by a similar response to a chemotactic attractant or by experimental manipulation soon segregate according to species (7, 8).

Later in development, pattern formation occurs, i.e. cells destined to become spores segregate from cells destined to become stalk cells. A model has been proposed (9) for this process which is based on the complementary interaction of contact-sensing cell surface macromolecules and the resulting effects of this interaction on the level of an intracellular "second messenger", perhaps cyclic adenosine 3',5'-monophosphate (cAMP). Predictions of this model that have since been confirmed include: 1) A break in a pseudoplasmodium should lead to two pseudoplasmodia each with normal pattern formation (10); 2) A step gradient of cAMP should be found along the pseudoplasmodium (11); and 3) Added plasma membranes should affect the development of cells (12, 13).

When normal cell contact cannot occur, development is blocked. Cells plated at low densities do not develop (14). Mutants that do not develop normally appear to be different antigenically on their cell surface than wild type cells (15, 16). When plasma membranes from aggregating cells are added to vegetative cells (13), development is inhibited. Similarly when pseudoplasmodial plasma membranes are added to disaggregated pseudoplasmodial cells, the

normal rapid resumption of morphogenesis is blocked (12). These two effects probably depend on the specific interactions of cell surface molecules since larger amounts of plasma membranes from earlier developmental stages or heterologous sources are required to produce the same results. The lectin, Concanavalin A (Con A) binds to many plasma membrane molecules in D. discoideum (17) and inhibits development (18, 19). This effect may be caused by the blocking or mimicking of normal cell contacts. The importance of cell contact in development is also indicated by the fact that pairs of aggregation deficient mutants have been isolated that can synergize to develop normally only when in contact (20).

The appearance of several developmentally controlled enzymatic activities depends upon normal cell contact. When pseudoplasmodia are disaggregated shortly before the scheduled appearance of an enzymatic activity and replated at cell densities too low for reaggregation, several enzymes never accumulate (21, 22). If reaggregation is allowed, each enzyme activity appears at the recapitulation of the normal morphological stage for that activity (22). In most cases, if the enzyme has already accumulated at the time of disaggregation, subsequent reaggregation still causes a new round of increase in enzymatic activity, and thus a supranormal enzymatic activity is attained (22). The enzymes referred to above are not associated with the plasma membrane. Recently, the activity of the plasma membrane associated enzyme cAMP-phosphodiesterase has been suggested to be controlled by cell contact by experiments in which

sensitive to different structural features.

III. Plasma Membrane Composition and Organization

Two different approaches have resulted in the characterization of cell surface molecules suggested to be involved in adhesion. A carbohydrate-binding protein, or lectin, has been isolated from D. discoideum cells (37). This lectin appears on the cell surface of aggregating cells (38) and therefore has been suggested to mediate adhesion by binding to its glycoconjugate on adjacent cells. However, the following facts suggest this lectin does not cause adhesion: 1) Although not found on the surface of vegetative cells grown on bacteria, it is found on the surface of axenic cells that are not cohesive (38, 39). 2) Antibody against the lectin does not inhibit aggregation (40).

By preparing antibodies against particulate preparations from aggregating cells and adsorbing the antibodies with vegetative particles, Beug et al. (6) have prepared an antibody that can block or reverse aggregation even when covering only a small percent of the cell surface (41). This immunologically defined aggregation site has been termed contact site A. No suggestion has been made that contact site A is a single protein. In fact, some aggregation-minus mutants partially adsorb contact site A activity suggesting it is directed against at least two antigenic sites (42).

Antigenic changes occur on D. discoideum cells as a result of development or a mutation that inhibits development. Spore cell specific and aggregation specific antigens have been detected (16,

43, 44). Antisera against wild type cells and some aggregation deficient mutants did not cross-react (15). Therefore, antigenic changes in the plasma membrane can be correlated with functional changes.

External plasma membrane proteins from vegetative cells have been studied by means of lactoperoxidase-catalyzed radioiodination followed by SDS gel electrophoresis (38, 45). Both groups found seven labeled bands over a similar molecular weight range. In addition, Smart and Hynes found that in developing cells, a newly labeled protein appears following aggregation. Disadvantages of this technique for determining cell surface proteins are that glycoproteins cannot be distinguished from polypeptides and that when a newly labeled component appears during development, one does not know if it was previously absent or cryptic.

A variety of lectins have been used to characterize carbohydrate-containing plasma membrane molecules. The most work has been done with Con A. Total Con A receptors increase in number but decrease in affinity during development of D. discoideum. Concomitantly, cells become less sensitive to Con A agglutination (19). Con A can induce the enzyme cyclic AMP phosphodiesterase (18). Recently, it has been shown that alkaline phosphatase, 5'-nucleotidase, and cAMP phosphodiesterase from vegetative plasma membranes bind to Con A columns. However, as development progresses a decreasing percent of the activity binds to the columns (46). Purified plasma membranes contain at least 40 species of Con A receptors

several of which change in amount during development (17). Another study detected at least 15 radioiodinable Con A receptor species (47).

Fewer studies have been done with other lectins. The endogenous lectin, discoidin, has been found to agglutinate glutaraldehyde fixed aggregating cells, but not fixed vegetative cells (48). Whether this phenomenon relates to normal aggregation is not clear since these experiments were done under conditions that allow nonspecific cell contacts (i.e. absence of EDTA). Different studies have found somewhat different agglutination activities for the lectins wheat germ agglutinin (WGA) and ricin. West et al. (1) find that WGA agglutinates cells throughout development while Reitherman et al. (48) find that only a small percent of aggregating cells can be agglutinated although 100% of vegetative cells are agglutinable. Ricin was found not to agglutinate cells at any stage of development[1] in one study, but was found to agglutinate a small percentage of aggregating cells in the other (48). The lectins soybean agglutinin and fucose-binding protein did not agglutinate cells at any stage.[1]

Studies on the spectrum of lectin receptor species in purified plasma membranes corroborate the results of West et al. on lectin agglutination. During development, a changing spectrum of WGA receptors are detectable. No ricin, soybean agglutinin, or fucose-binding protein receptors are detectable at any develop-

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[1]West, C. M., McMahon, D., and Molday, R. S., submitted for publication.

mental stage.[1]

Lectin studies have also yielded information on plasma membrane organization. Con A receptors are capped by the addition of Con A (49, 50), a process that may facilitate agglutination (19). Following the capping of Con A receptors, WGA receptors are still detected uniformly on the cell surface, showing that Con A and WGA receptors are at least partially independent, both in composition and mobility (50). During the capping process, Con A receptors appear to first disappear from microvilli before disappearing from the rest of the cell surface as visualized in the scanning electron microscope with Con A conjugated to macrospheres (50). This suggests that plasma membrane organization may be different on microvilli than elsewhere on the cell surface.

Intramembranous particles have been observed in the Dictyostelium plasma membrane (51). The size of these particles increases during development or in response to added cAMP (52). The mechanism or significance of this effect is not currently understood.

In this thesis, I examine many aspects of plasma membrane composition and organization and their relation to function. I have tried to answer the following questions: Chapter 1) How do plasma membrane composition and topography change during normal development? Chapters 2 and 3) How are the normal changes in composition and topography perturbed by the inhibition of development? Chapter 4) Can defects in the plasma membrane be detected before the phenotypic expression of a mutation blocking development?

Chapter 5) Is it feasible to add a glycoprotein purified from the plasma membrane of one strain of cells into the plasma membrane of another strain of cells? The answers I have found to these questions indicate that several plasma membrane features are associated with cells undergoing or capable of undergoing normal development and that it is feasible to add purified plasma membrane components to cells.

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CHAPTER I

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

DEVELOPMENTAL AND TOPOGRAPHIC ANALYSIS OF POLYPEPTIDE AND GLYCOPROTEIN COMPOSITION

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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 × *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 × *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-

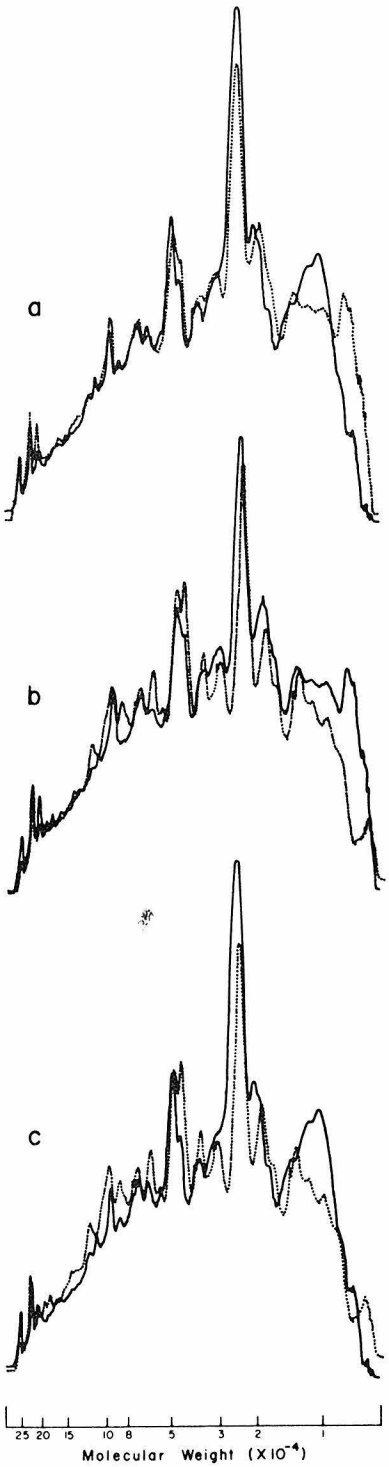

Fig. 1. Alterations of plasma membrane polypeptides during development. Densitometric scans of gels prepared by the method of Fairbanks et al. [24] and stained with Coomassie brilliant blue are superimposed to illustrate changes in polypeptide composition of the plasma membrane. (a) Log phase (solid 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).

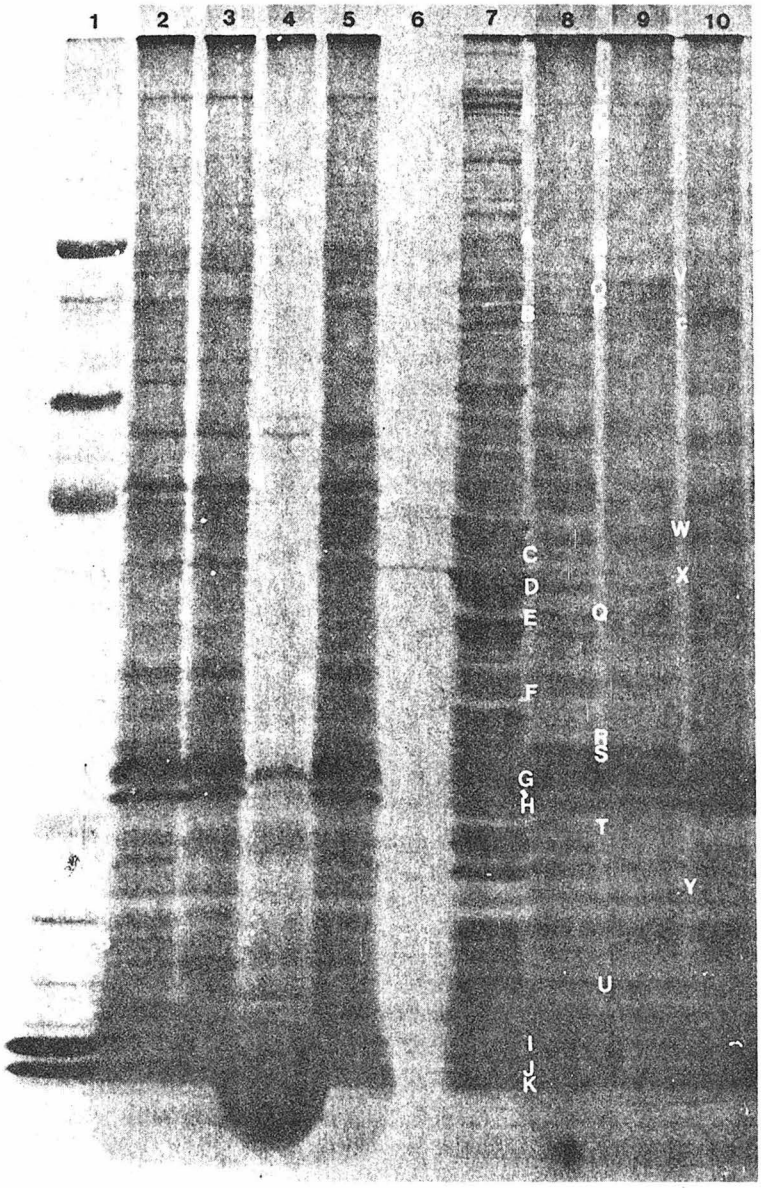

Fig. 2. Exponential gradient gel electrophoresis of polypeptides. Lanes from left to right are: (1) standard proteins as described in Materials and Methods; (2) plasma membranes from pre-culmination cells; (3) plasma membranes from pronase-treated pre-culmination cells; (4) pronase-treated plasma membranes 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

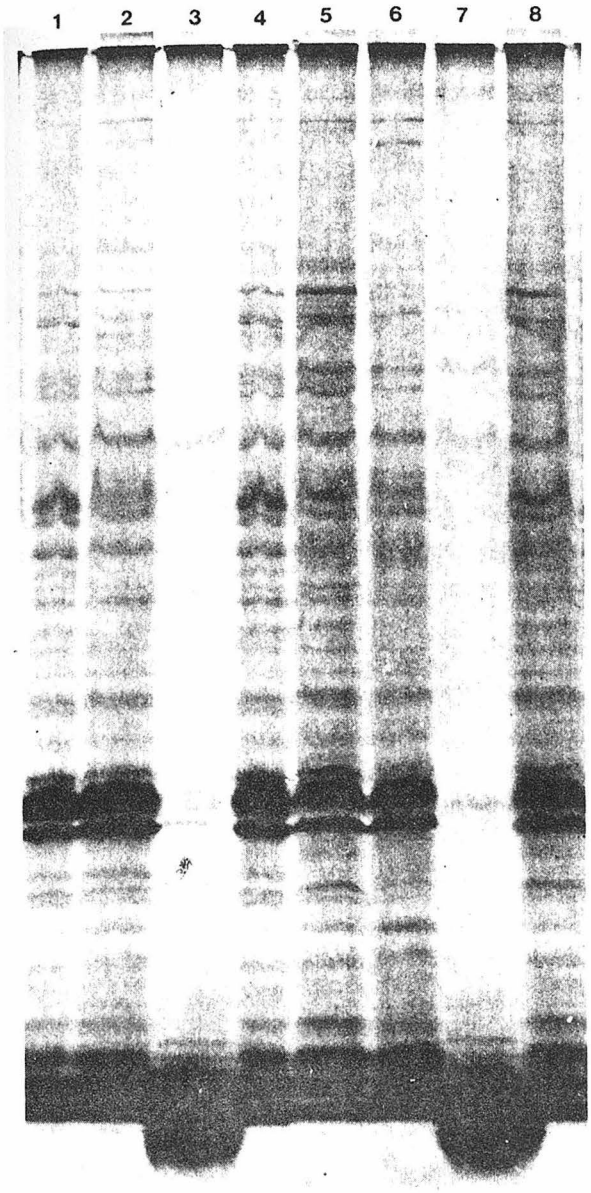

Fig. 3. Exponential gradient gel electrophoresis of polypeptides from log and aggregation phase plasma membranes. Lanes are from left to right: (1) log phase plasma membranes; (2) plasma membranes from pronase-treated log phase cells; (3) pronase-treated plasma membranes from log phase cells; (4) as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

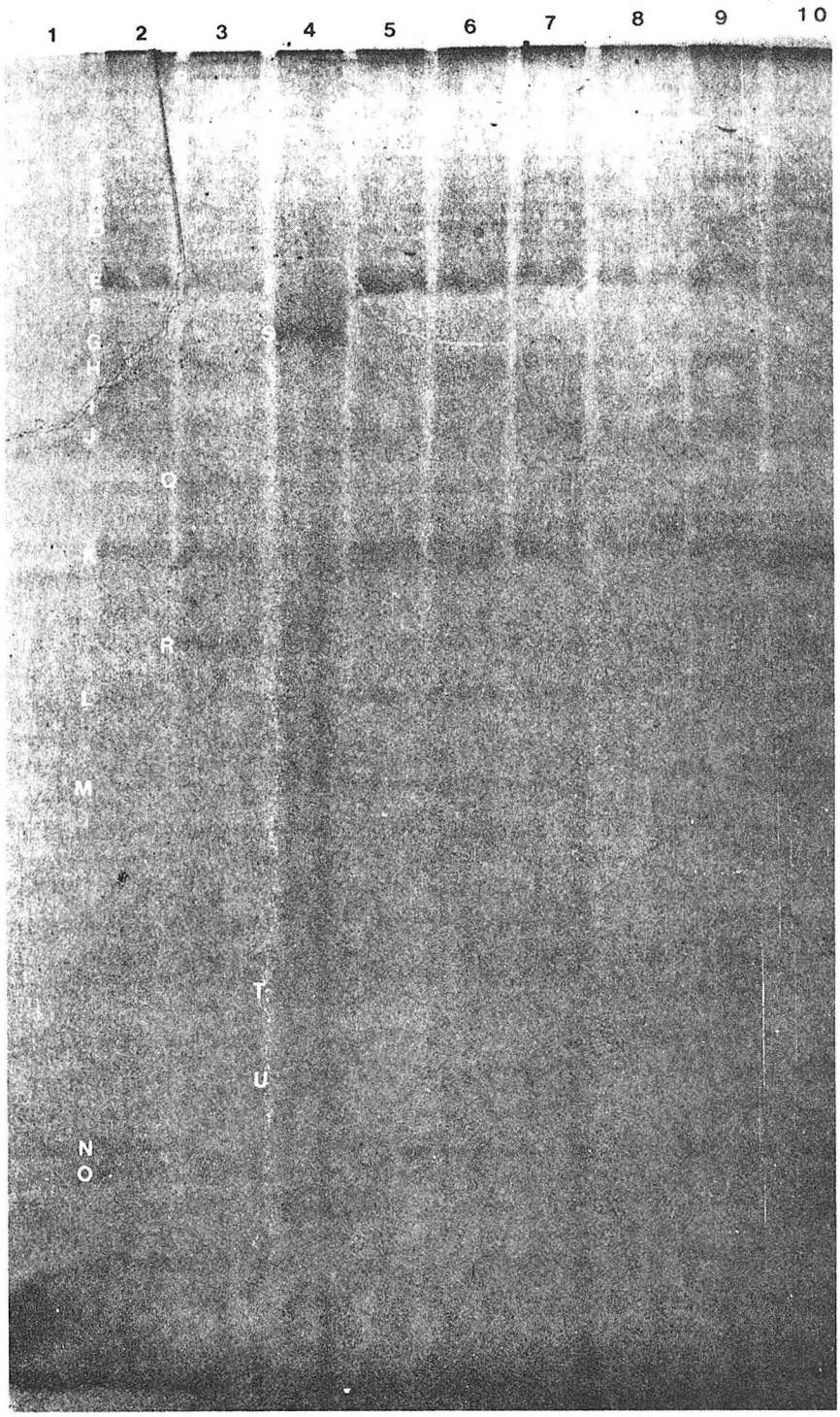

Fig. 4. Exponential gradient gel electrophoresis of glycoproteins. Lanes from left to right are: (1) total protein from log phase amoebae; (2) plasma membranes from log phase amoebae; (3) plasma membranes from aggregation phase cells; (4) plasma membranes from preculmination phase cells; (5) as in 2; (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.

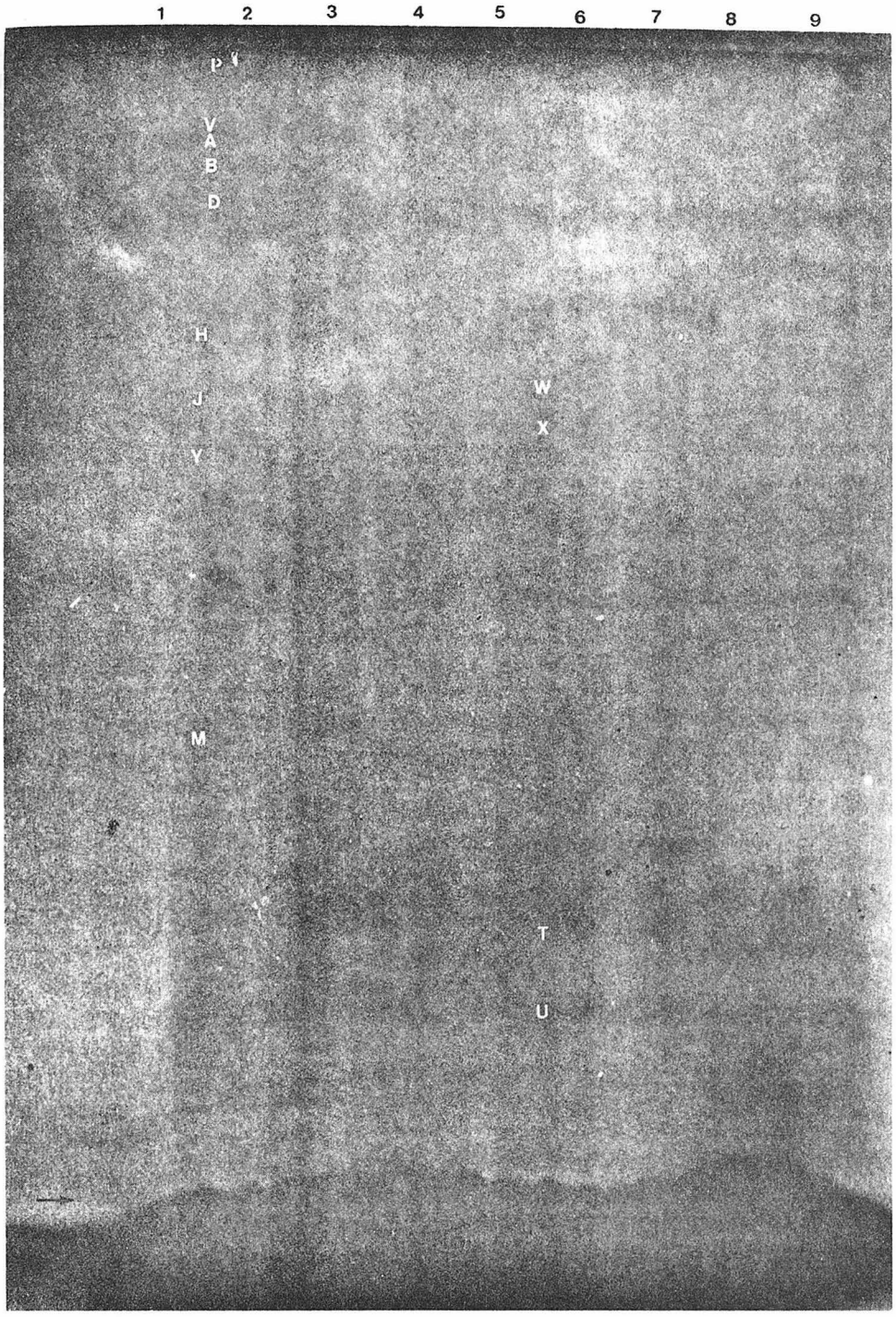

Fig. 5. Exponential gradient gel electrophoresis of glycoproteins from aggregation and pre-culmination stage plasma membranes. Lanes from left to right are: (1) phosphorylase *b*, glucose oxidase, avidin, cytochrome *c* (2.5 μg each), arrows mark the position of phosphorylase *b* and cytochrome *c*; (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.

Acknowledgements

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CHAPTER II

THE EFFECTS OF INHIBITION OF DEVELOPMENT ON CHANGES IN PLASMA MEMBRANE COMPOSITION AND TOPOGRAPHY

THE EFFECTS OF INHIBITION OF DEVELOPMENT ON CHANGES IN PLASMA MEMBRANE COMPOSITION AND TOPOGRAPHY[1]

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(Running Title: Inhibition of Developmental Changes in the Plasma Membrane)

[1]This is paper No. 5 in a series entitled "The Role of the Plasma Membrane in the Development of Dictyostelium discoideum". Paper No. 4 is Reference 8.

SUMMARY

Previous experiments have demonstrated that during the development of the cellular slime mold, Dictyostelium discoideum, the macromolecular composition of the plasma membrane changes dramatically. In addition the exposure on the external face of the plasma membrane of some developmentally conserved polypeptides varies during development. When development and differentiation were prevented with cycloheximide or mutation the bulk of the changes in composition were prevented. These treatments also prevented the disappearance of macromolecules and were particularly effective in preventing changes of glycoprotein composition. Similarly the program of developmental changes of exposure of polypeptide was disrupted by both treatments. Cycloheximide, but not the mutation, stimulated the premature disappearance of six external polypeptides. This may reflect a rapid turnover of these polypeptides, which cannot be replaced in the absence of protein synthesis.

INTRODUCTION

The biological functions of the cell surface of Dictyostelium change drastically during development and concurrently the plasma membrane changes in many ways (1). Changes of plasma membrane components include changes in antigens (2-4), macromolecules exposed at the exterior cell surface (5,6), and lectin receptors (7-9). Cell surface components that change in amount include contact sites A (10), discoidin (11) (protein S in ref. 6), cAMP receptor (12-14), and cAMP phosphodiesterase (15,16).

At least 28 of 65 plasma membrane polypeptides change significantly in amount present during development from vegetative amoebae to pseudoplasmodium (6). Eleven of these 28 polypeptides are located on the cell surface. Six other polypeptides change in degree of exposure at the surface (as indicated by sensitivity to pronase) at various times during development (6). An almost complete change of glycoproteins occurs. Twenty-four of twenty-five glycoprotein species change in amount present during development from amoeba to pseudoplasmodium (6).

One way to assess the relationship between changes in the cell surface and morphogenesis and cellular differentiation is to attempt to uncouple the two processes by inhibiting development. This can be done with developmental mutants. For example, Beug et al. (4) found that five of seven aggregation deficient mutants which they tested could adsorb aggregation inhibiting antibodies. Therefore,

the appearance of the contact site A antigen, which mediates aggregation-specific cell cohesion, was not prevented in most of these mutants. Development may also be blocked with inhibitors. In this paper, both mutation and cycloheximide have been used to examine the dependence of changes in the spectrum of plasma membrane macromolecular composition on normal morphogenesis. Our experiments were designed to answer the following questions: 1) Are the changes in the spectrum of macromolecules in the plasma membrane tightly coupled to the normal sequence of development or are they the result of the conditions necessary to initiate development? 2) Do these changes (particularly the disappearance of macromolecules) occur when protein synthesis is inhibited?

Since the effects of inhibition of development on many of the biological functions of the plasma membrane are known, analysis of the program of plasma membrane changes under similar conditions should help identify prospective molecules involved in the processes of cell adhesion, pattern formation, etc.

MATERIALS AND METHODS

Cells

Cells used in this work were D. discoideum strains A3 (ref. 17, page 13) and HM 2, a spontaneous aggregation minus mutant (isolated by D. M. from strain A3).

Development

Cells were cultured, harvested, and incubated under the

conditions previously described (6,18). When the development of A3 cells in suspension was inhibited with cycloheximide, 500 µg/ml of the drug was routinely added to the aggregation buffer (19) and prevented formation of contact sites A and B. However, when cells were incubated on filters soaked with 50% diluted lower pad solution (20) + 500 µg/ml (or 1 mg/ml) cycloheximide, development was only partially inhibited. Therefore, we used 2 mg/ml cycloheximide to inhibit the development of cells on filters. Cycloheximide treatments of 500 µg/ml for cells in suspension or 2 mg/ml for cells on filters each inhibit protein synthesis at least 95% and are equivalent drug doses on a per cell basis.

Contact sites A and B were defined by the method of Beug et al. (10) using 10 mM EDTA.

Preparation and analysis of samples

Membrane preparation, pronase treatments and gel electrophoresis were as previously described (6,18,21). Cycloheximide-treated A3 cells at 18 h on filters were not pronase-treated because of difficulty in collecting sufficient material. The results shown are gels representative of the results of two or three experiments. The designation, (gp), is occasionally used to identify glycoproteins in the text to prevent ambiguity.

Assay of protein synthesis

Cells were washed and resuspended as described above for development. The resuspension buffers contained 1 mM methionine and 2 µCi/ml ^{3}H-methionine. One ml samples of cells (10^{7} cells/ml) were

harvested at intervals and added to cold 10% trichloroacetic acid. Cells (0.1 ml of 10^8 cells/ml) that had been plated onto 25 mm HABG Millipore filters resting on pads saturated with 0.5 ml of the resuspension solution were harvested at intervals by washing the cells off the filters with cold H_2O delivered by a Pasteur pipette. An equal volume of cold 10% trichloroacetic acid was added. All samples were prepared for counting as described (22) except that the 10% trichloroacetic acid wash contained 10 mM methionine.

RESULTS

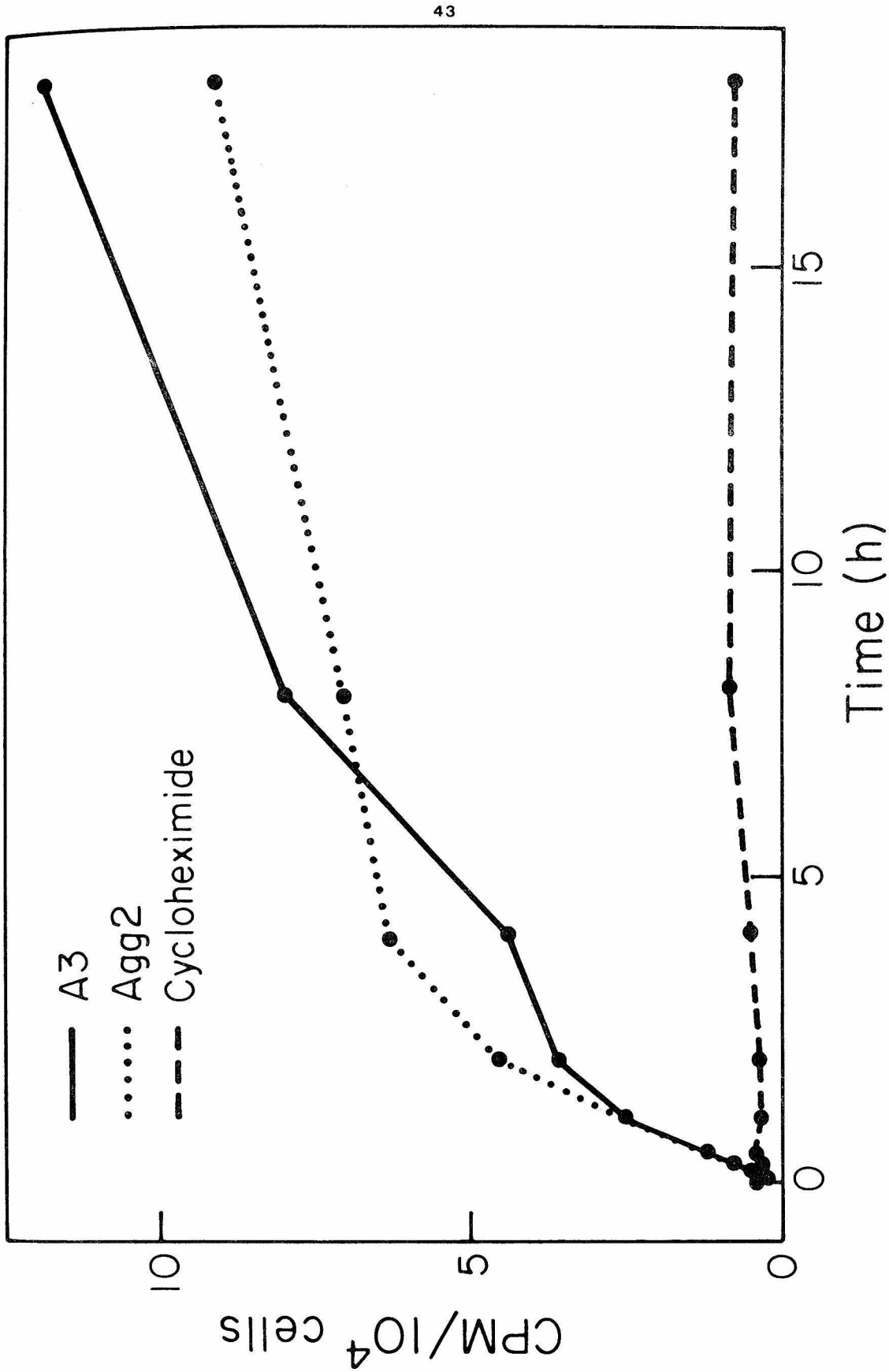
Protein Synthesis

We measured the synthesis of protein in untreated and cycloheximide-treated A3 cells and in cells of the mutant, HM 2 (Fig. 1). Cycloheximide treatment of A3 cells on filters (2 mg/ml) (Fig. 1) or in suspension (500 µg/ml) (data not shown) cause an immediate inhibition of protein synthesis of at least 95%. Protein synthesis in the mutant is similar to that in wild type cells. Late in the experiment the mutant appears to be slightly deficient in protein synthesis. However, if the amount protein synthesis is calculated on the basis of total cellular protein, this difference disappears.

Development of Cells

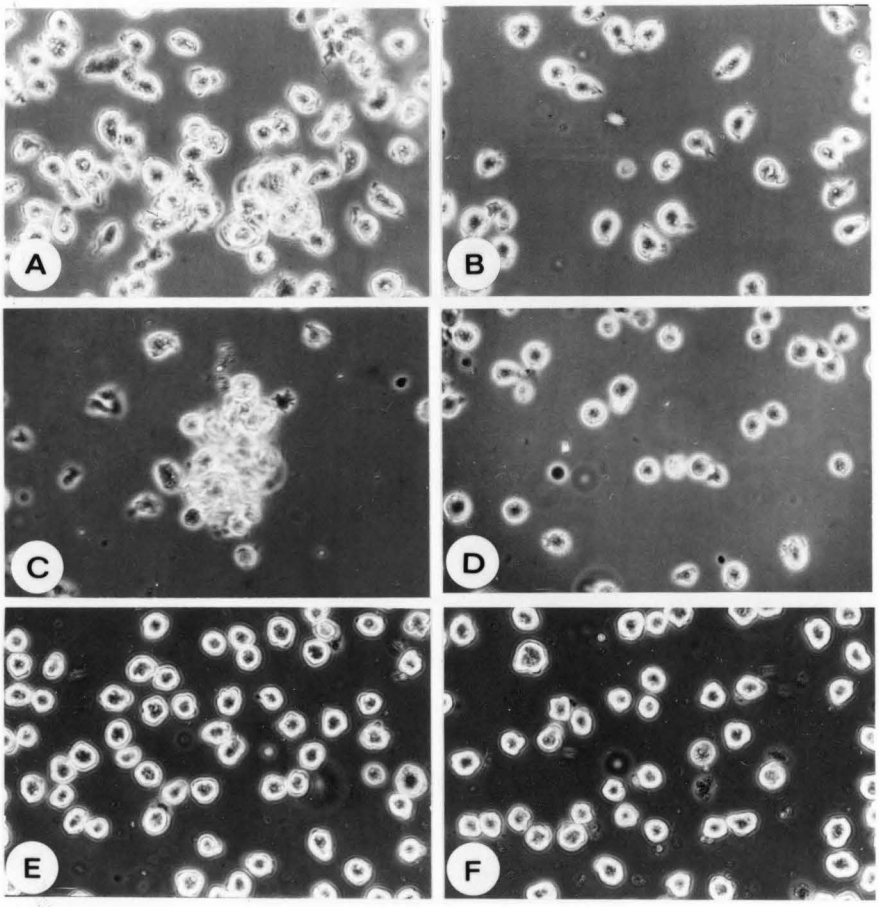
The plasma membrane composition of two different developmental stages of wild type A3 cells and cells of the aggregation minus mutant HM 2 or cycloheximide inhibited A3 cells have been compared. These

FIGURE 1. Comparative protein synthesis during incubation on filters for A3, HM 2, and cycloheximide treated (2 mg/ml) A3 cells. Protein synthesis was assayed as described in the Methods.


A3
Agg2
Cycloheximide
CPM/10⁴ cells
0
5
10
0
5
10
15
Time (h)

stages are cultures incubated in suspension for 12 h or for 18 h on filters. For simplicity, we will occasionally refer to all 12 h suspension cultures as early aggregation and all 18 h filter cultures as preculmination even when normal development has been inhibited. Early aggregation A3 cells are developmentally equivalent to cells incubated on filters for approximately 5 h. They have not formed contact sites A (10) but if replated onto filters they complete development about 5 h before vegetative cells plated at the same time. Early aggregation HM 2 cells have also not formed contact sites A, but unlike A3 will not undergo any further visible development when replated on filters. In suspension, early aggregation A3 and HM 2 cells are in aggregates mediated by contact sites B (10). In contrast, cycloheximide treated early aggregation cells are found to be completely single cells and thus must lack both contact sites A and B (Fig. 2). A3 preculmination cells have completed aggregation and are undergoing the morphogenetic movements that lead to culmination. Similarly incubated HM 2 cells and cycloheximide treated cells show no visible signs of development (23, manuscript in preparation). Lodish et al. showed that the pattern of whole cell protein synthesis in HM 2 cells incubated under normal conditions for development never varies from that associated with the 0 to 6 h stage of development in wild type cells (24). In addition no detectable synthesis of four developmentally controlled enzymes: tyrosine transaminase, UDPG pyrophosphorylase, glycogen phosphorylase and alkaline phosphatase was detected in HM 2 (D.M., unpublished results). Therefore the HM 2 mutation probably acts very early in development.

FIGURE 2. Early aggregation phase cells. A3 (A,B), HM 2 (C,D) and cycloheximide treated A3 cells (E,F) were shaken for 12 h in aggregation buffer (18) as described in the Methods. Aliquots were then photographed in the presence (B,D,F) or absence (A,C,E) of 10 mM EDTA.


A
B
C
D
E
F

Correlation of Membrane Composition with Development

Comparisons of the polypeptide and glycoprotein composition of plasma membranes isolated from wild type and inhibited cells are presented in Figs. 3-9. We have presented only the major developmental changes in plasma membrane macromolecular composition because these can be studied with more certainty. Four differences which appear in Figs. 3-6 but are not flagged were not reproducible. In order to make the data easier to assimilate, the results were organized into Tables 1-4.

A polypeptide provides an example of how the tables and illustrations of gels were prepared. Polypeptide M is absent from vegetative A3 plasma membranes but appears in early aggregation cells (6). This is also evident from a comparison of lanes 1 and 6 in Fig. 3. Plasma membranes from cycloheximide-treated early aggregation cells (Fig. 3, lane 5) do not contain polypeptide M. This difference has been flagged with the letter M between lanes 5 and 6. Early aggregation HM 2 plasma membranes contain M in an amount similar to that found in A3 (Fig. 3, lane 7). In Table 1, in the category of early aggregation increases, we have placed polypeptide M in the category of components whose increase is cycloheximide-sensitive. Polypeptide M normally disappears from the plasma membrane of preculmination phase cells (6). This decrease does not occur in HM 2 cells (Fig. 4, lane 3) so it is included in Table 1 as a decrease blocked by mutation. Since the increase in the amount of M does not occur in early aggregation cells treated with cycloheximide, the present experiments do not allow us to determine whether cycloheximide prevents its disappear-

FIGURE 3. Exponential gradient gel electrophoresis of plasma membrane polypeptide from vegetative and early aggregation cells. Lanes from left to right are:

1) Plasma membranes from vegetative A3 cells;
2) Plasma membranes from cycloheximide treated early aggregation A3 cells;
3) Plasma membranes from pronase treated cycloheximide treated early aggregation A3 cells;
4) Pronase treated plasma membranes from cycloheximide treated early aggregation A3 cells;
5) As in 2;
6) Plasma membranes from early aggregation A3 cells;
7) Plasma membranes from early aggregation HM 2 cells;
8) Plasma membranes from pronase treated early aggregation HM 2 cells;
9) Pronase treated plasma membranes from early aggregation HM 2 cells.

Symbols on the gels indicate the identity of polypeptides. Symbols between lanes flag differences between those lanes as described in the text. Arrows indicate the positions of bands that are deficient in pronase treated isolated HM 2 plasma membranes.

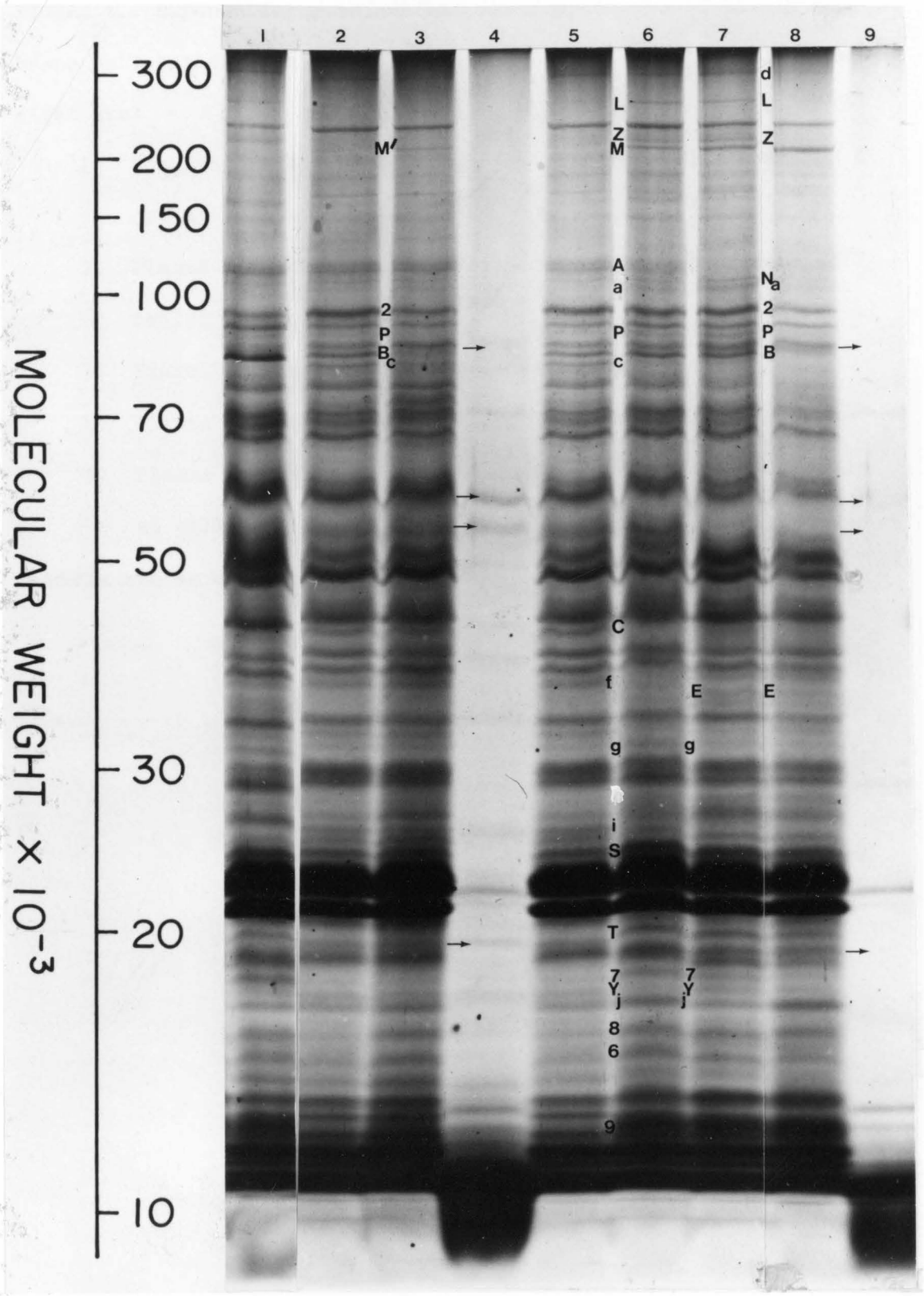

1
2
3
4
5
6
7
8
9
300
200
150
100
70
50
30
20
10
MOLECULAR WEIGHT × 10⁻³
M′
2
P
B
c
L
Z
M
A
a
P
c
C
f
g
i
S
T
7
Y
j
8
6
9
E
g
7
Y
j
d
L
Z
N
a
2
P
B
E

FIGURE 4. Exponential gradient gel electrophoresis of plasma membrane polypeptides from preculmination cells. Lanes from left to right are:

1) Pronase treated plasma membranes from preculmination HM 2 cells;
2) Plasma membranes from pronase treated preculmination HM 2 cells;
3) Plasma membranes from preculmination HM 2 cells;
4) Plasma membranes from preculmination A3 cells;
5) Plasma membranes from cycloheximide treated preculmination A3 cells.

Symbols are as described for Fig. 3.

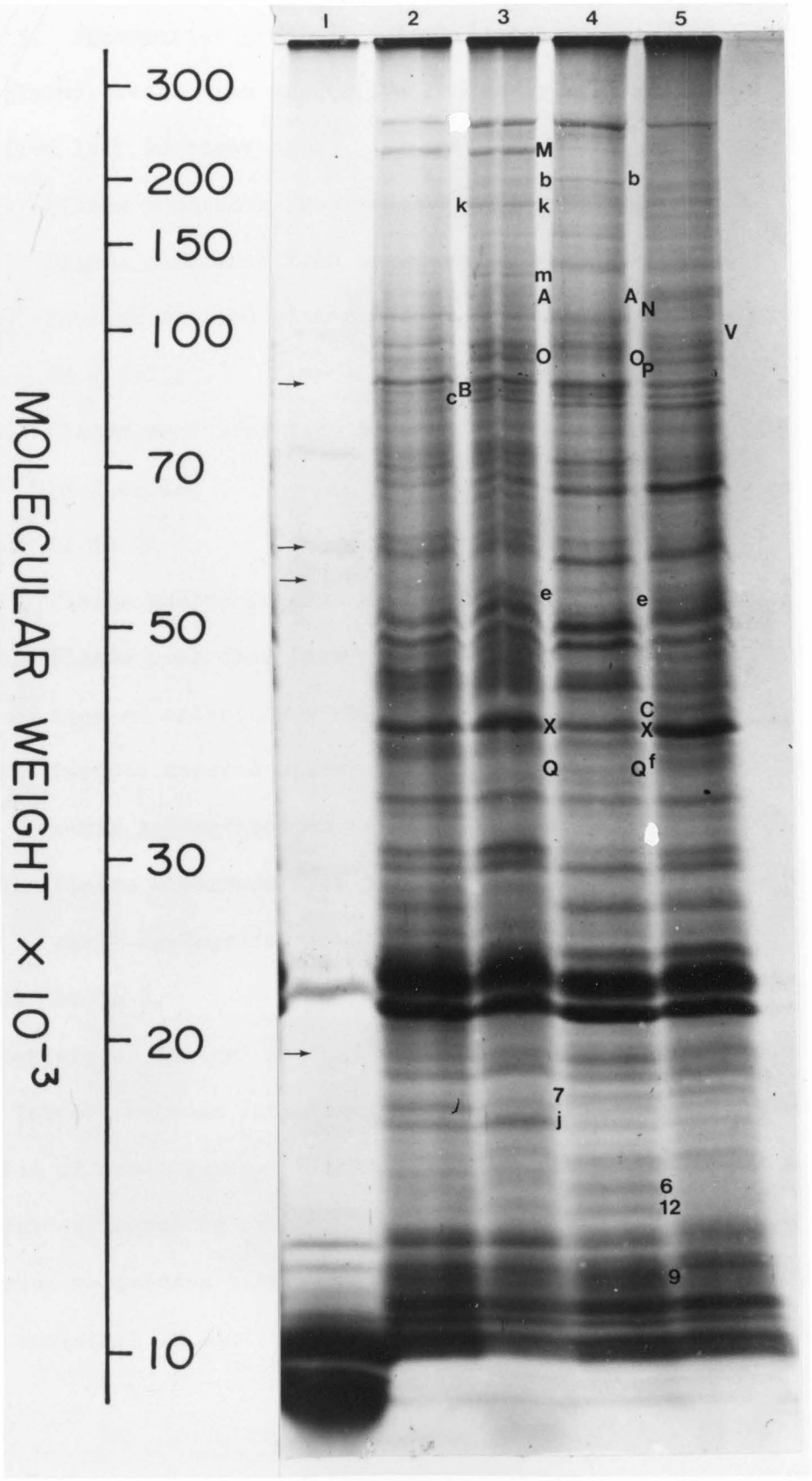

1
2
3
4
5
300
200
150
100
70
50
30
20
10
MOLECULAR WEIGHT × 10⁻³
M
b
k
k
b
m
A
A
N
V
O
O
P
c
B
e
e
C
X
X
Q
Q
f
7
j
6
12
9

FIGURE 5. Exponential gradient gel electrophoresis of plasma membrane glycoproteins from vegetative and early aggregation cells. Lanes from left to right are:

1) Plasma membranes from vegetative A3 cells;
2) Plasma membranes from early aggregation HM 2 cells;
3) Pronase treated plasma membranes from early aggregation HM 2 cells;
4) Plasma membranes from pronase treated early aggregation HM 2 cells;
5) As in 2;
6) Plasma membranes from early aggregation A3 cells;
7) Plasma membranes from cycloheximide treated early aggregation A3 cells;
8) Pronase treated plasma membranes from cycloheximide treated early aggregation A3 cells;
9) Plasma membranes from pronase treated cycloheximide treated early aggregation A3 cells;
10) As in 7.

Letters on the gel indicate the identity of glycoproteins. White letters between lanes indicate differences caused by the inhibition of development. Black letters between lanes indicate differences caused by pronase treatment. Of the glycoproteins sensitive to pronase treatment of isolated plasma membrane, only those resistant in similarly treated A3 plasma membrane are flagged.

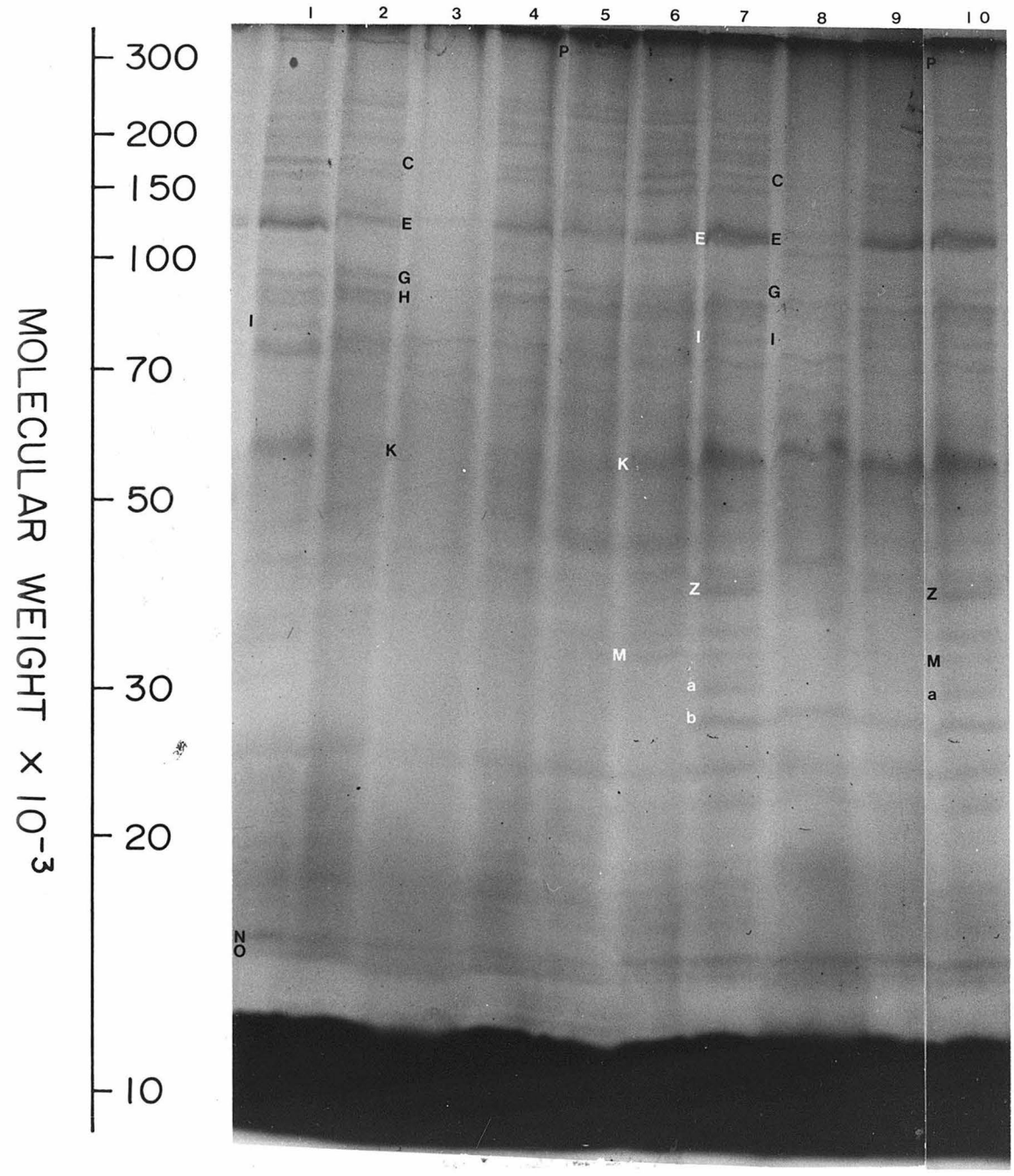

1
2
3
4
5
6
7
8
9
10
300
200
150
100
70
50
30
20
10
MOLECULAR WEIGHT X 10⁻³
P
C
E
G
H
I
K
M
Z
a
b
N
O

FIGURE 6. Exponential gradient gel electrophoresis of plasma membrane glycoproteins from preculmination cells. Lanes from left to right are:

1) Plasma membranes from cycloheximide treated preculmination A3 cells;
2) Plasma membranes from preculmination A3 cells;
3) Plasma membranes from preculmination HM 2 cells;
4) Plasma membranes from pronase treated preculmination HM 2 cells;
5) Pronase treated plasma membranes from preculmination HM 2 cells.

Symbols are as in Figure 5.

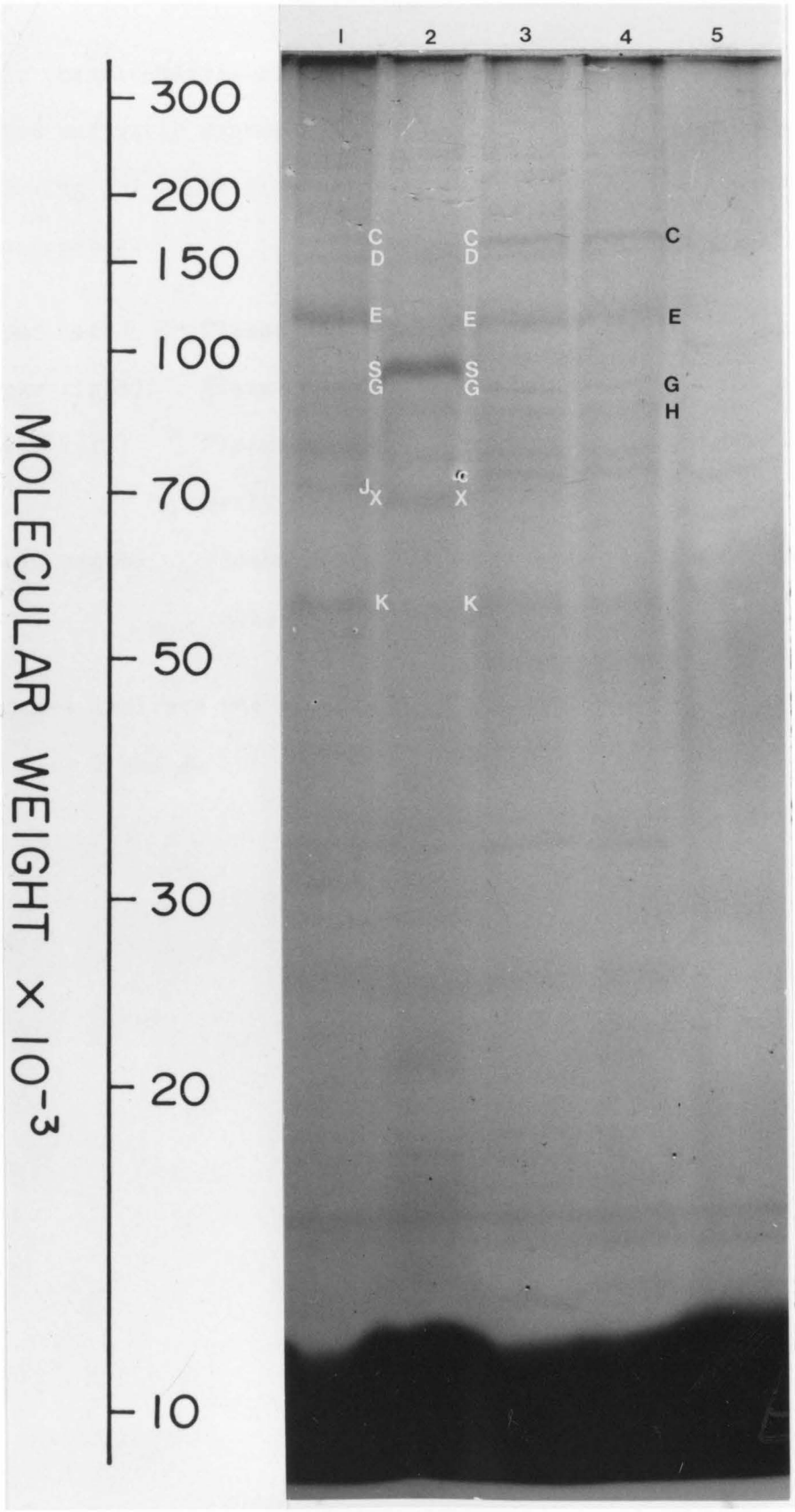

1
2
3
4
5
300
200
150
100
70
50
30
20
10
MOLECULAR WEIGHT × 10⁻³
C
D
E
S
G
J
X
K
C
D
E
S
G
X
K
C
E
G
H

FIGURE 7. Densitometric scans of plasma membrane glycoproteins from vegetative and early aggregation cells. Photographic negatives of the following gel lanes from Figure 5 were scanned on a Syntex AD-1 Autodensitometer:

Upper left) Plasma membranes from vegetative A3 cells;

Upper right) Plasma membranes from early aggregation A3 cells;

Lower left) Plasma membranes from cycloheximide treated early aggregation A3 cells;

Lower right) Plasma membranes from early aggregation HM 2 cells.

Letters indicate the positions of glycoproteins noted in Figure 5 and Tables 1 and 2.

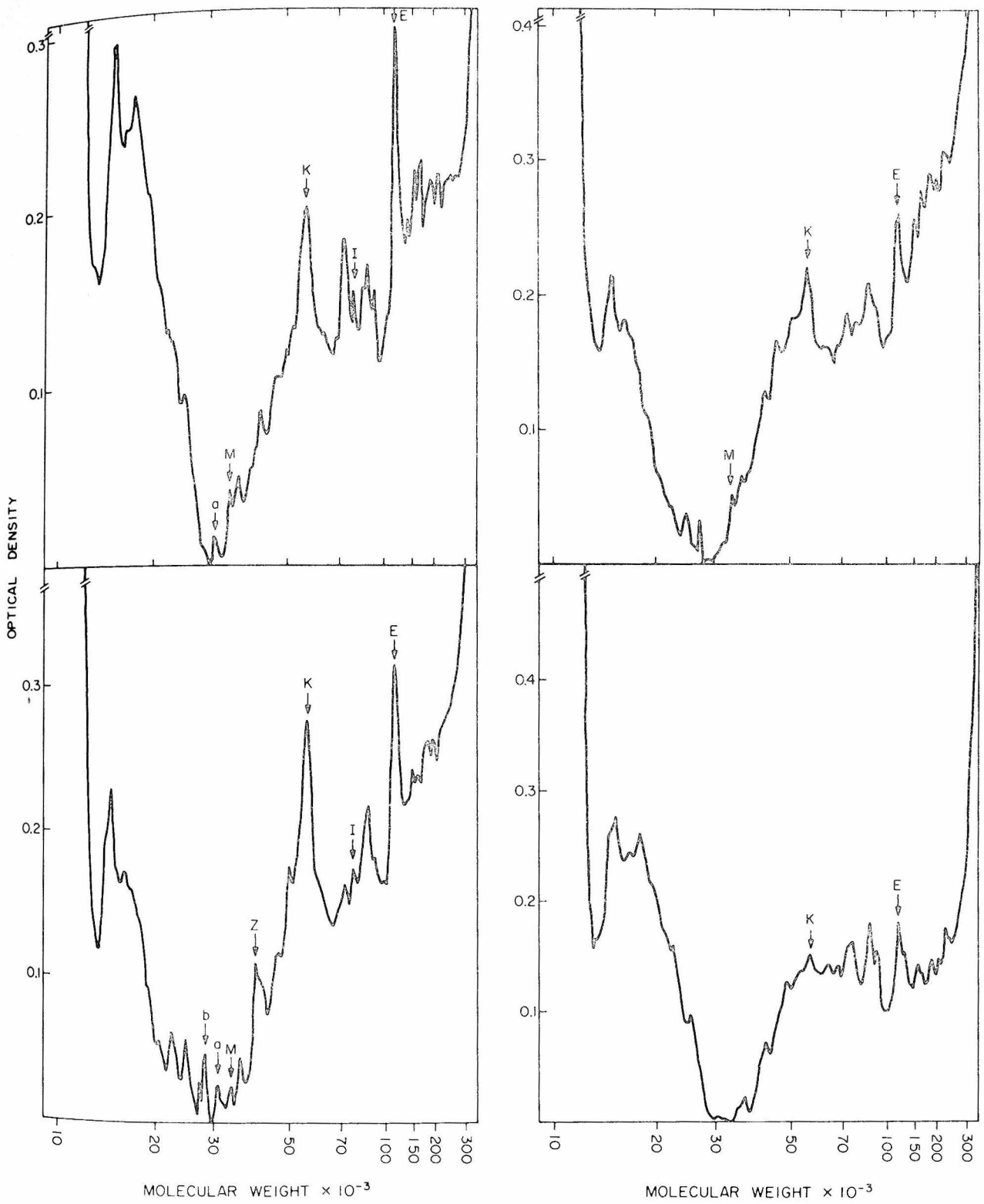

OPTICAL DENSITY
0.3
0.2
0.1
E
K
I
M
a
0.4
0.3
0.2
0.1
E
K
M
0.3
0.2
0.1
E
K
I
Z
b
a
M
0.4
0.3
0.2
0.1
E
K
10
20
30
50
70
100
150
200
300
MOLECULAR WEIGHT × 10⁻³
MOLECULAR WEIGHT × 10⁻³

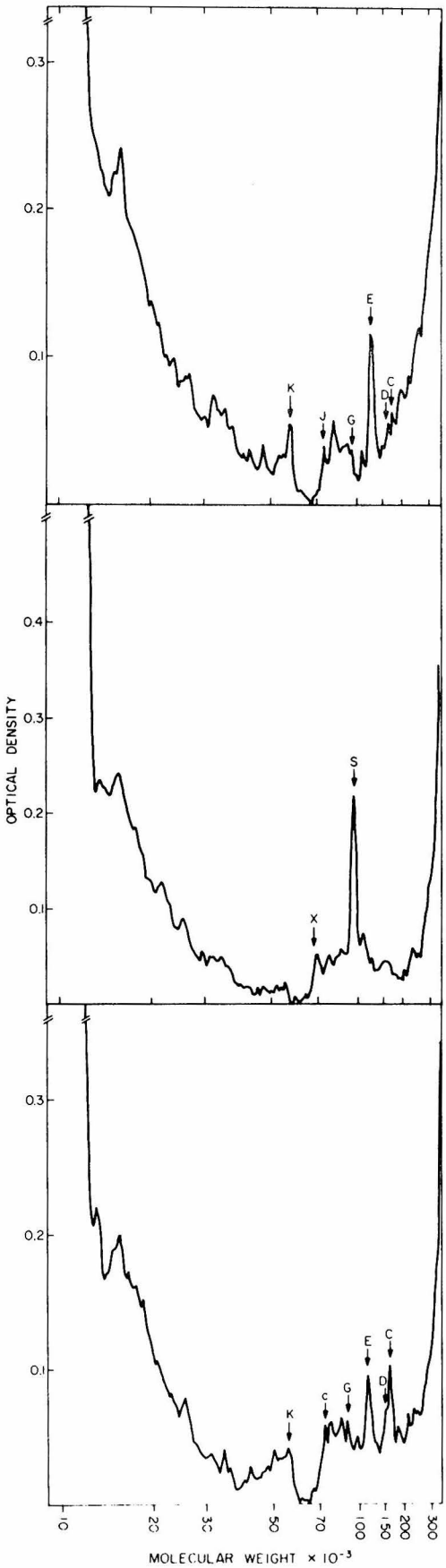
FIGURE 8. Densitometric scans of plasma membrane glycoproteins from preculmination cells. Photographic negatives of the following gel lanes from Figure 6 were scanned on a Syntex AD-1 Autodensitometer:

Top) Plasma membranes from cycloheximide treated A3 preculmination cells;

Middle) Plasma membranes from A3 preculmination cells;

Bottom) Plasma membranes from HM 2 preculmination cells.

Letters indicate the position of glycoproteins noted in Fig. 6 and Tables 1 and 2.


OPTICAL DENSITY
0.3
0.2
0.1
K
J
G
E
D
C
0.4
0.3
0.2
0.1
X
S
0.3
0.2
0.1
K
c
G
E
D
C
10
20
30
50
70
100
150
200
300
MOLECULAR WEIGHT × 10⁻³

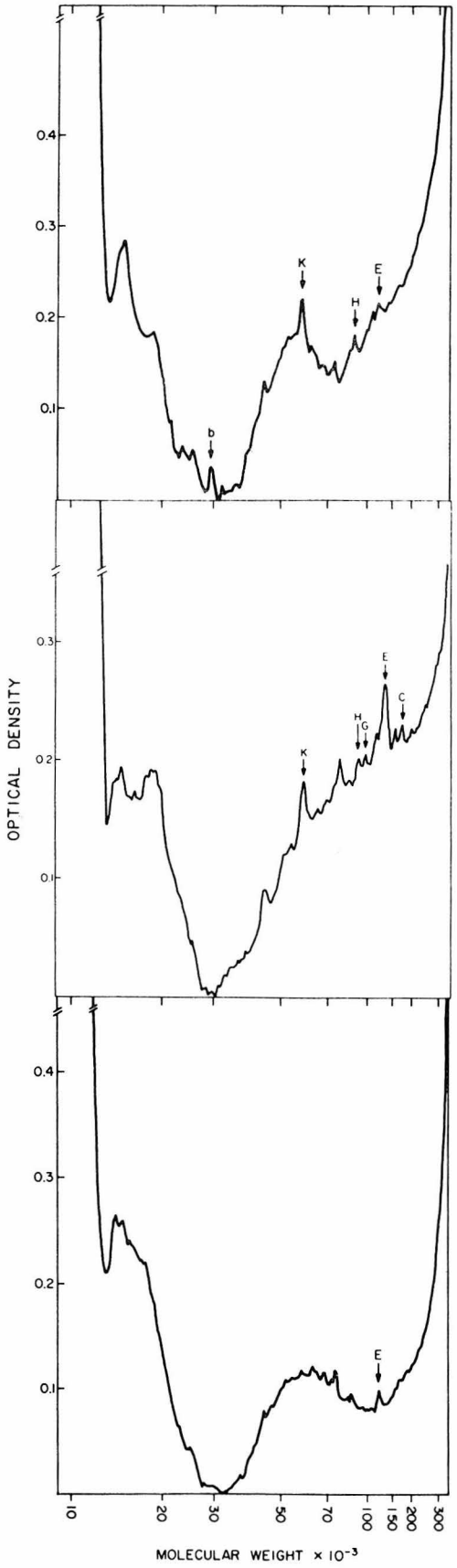

OPTICAL DENSITY
0.4
0.3
0.2
0.1
b
K
H
E
0.3
0.2
0.1
K
H
G
E
C
0.4
0.3
0.2
0.1
E
10
20
30
50
70
100
150
200
300
MOLECULAR WEIGHT × 10⁻³

TABLE 1. Normal Developmental Changes Blocked by Cycloheximide and/or Mutation. This table was compiled from Figures 3-8 as described in the text. * - The normal change occurs prematurely in cycloheximide treated cells. All of these molecules are found on the exterior surface of the plasma membrane (6).

Table 1

Normal Developmental Changes Blocked by Cycloheximide and/or Mutation

Treatment	Early Aggregation		Preculmination	
	Decreases	Increases	Decreases	Increases
a) Polypeptides				
Cycloheximide	A,C	M,a	A,M(?),N,O,P,e	Q,b
Mutation	E	---	A,M,O,e	Q,b
Molecules that normally show the change	A,B,C,E	M,a	A,L*,M,N,O,P, S*,T*,Z*,d,e	Q,V,b
b) Glycoproteins				
Cycloheximide	E,I,a	---	C,D,E,G,J,K	S,X
Mutation	J(?)	---	C,D,E,G,K	S,X
Molecules that normally show the change	E,I,J,a	---	C,D,E,G,J,K,N, O,P	S,X

ance at preculmination, so we have put M (?) in Table 1 in the section containing cycloheximide-sensitive decreases of polypeptides in preculmination phase cells. Polypeptide M and glycoprotein J which is already reduced in amount in vegetative HM 2 plasma membranes are the only such unknowns in Table 1.

The data summarized in Table 1 can be analyzed in several ways. Table 1 shows that of 33 developmental changes in the amount of polypeptides or glycoproteins, 6 are unaffected by either treatment, 10 are inhibited only by cycloheximide, and 12 are inhibited by both cycloheximide treatment and mutation. Only one change is allowed in the presence of cycloheximide but inhibited by mutation. In addition, four polypeptides which normally decrease at preculmination in untreated or mutant cells, decrease precociously by early aggregation in the presence of cycloheximide.

Changes of the amounts of glycoproteins were slightly more dependent on normal development than changes of amount of polypeptides. Most changes which occur before early aggregation are inhibited only by cycloheximide, suggesting that the mutant can make some preparations for development which can, however, be blocked by cycloheximide.

As might be expected, almost all increases in the amount of macromolecules which occur during development are prevented by inhibition of development. Although decreases in amounts of macromolecules are less dependent on normal development, the majority of these are also inhibited by cycloheximide or mutation.

Aberrant Changes in Plasma Membrane Macromolecular Composition

When development is disrupted, differences in plasma membrane composition between wild type and inhibited cells are apparent that are not blocked developmental changes. These aberrant changes are summarized in Table 2.

One major class of these changes is the disappearance of polypeptides in the presence of cycloheximide which are normally exposed on the exterior face of the plasma membrane. Six of thirteen polypeptides which were previously found (6) to be at the cell surface of early aggregation or preculmination A3 cells decrease in amount or disappear from the plasma membranes of cycloheximide-treated cells but remain in the membrane of the mutant cells. An attractive explanation for this abnormal disappearance is that polypeptides on the outer face of the plasma membrane normally turn over rapidly but cannot be replaced when protein synthesis is inhibited.

Cycloheximide treatment causes the loss of six low molecular weight polypeptides on the interior face of the plasma membrane. Two of these are also lost in mutant cells. Four novel polypeptides appear in inhibited cells. Two polypeptides (Y, c) appear to increase prematurely in inhibited cells. Since, in the case of an aberrant appearance, our only criterion for identity of these polypeptides with the polypeptides found at a later stage in wild type cells is their molecular weight, independent evidence of the identities of these polypeptides is required. There are relatively few aberrant changes in glycoprotein composition when development is inhibited (Table 2B).

TABLE 2. Aberrant Differences in Plasma Membrane Composition Between Wild Type and Inhibited Cells. This table summarizes differences in plasma membrane composition between wild type and inhibited cells that cannot be explained by blocked developmental changes. Circled bands are novel to that treatment. Underlined bands are cell surface polypeptides removed by cycloheximide treatment.

Table 2

Aberrant Differences in Plasma Membrane Composition Between Wild Type and Inhibited Cells

	Early aggregation		Preculmination	
Treatment	Decrease	Increase	Decrease	Increase
a) Polypeptide				
Cycloheximide	L,P,S,T,Z g,j,6,7,8,9	Y,c,(f), i	6,9,12	X, (f)
Mutation	g,j,7	Y	7	X,j,(k),(m)
b) Glycoproteins				
Cycloheximide	—	Z,(b)	—	—
Mutation	K,M	—	—	C,c

Comparative Pronase Sensitivity of Plasma Membrane Macromolecules from Normal and Inhibited Cells

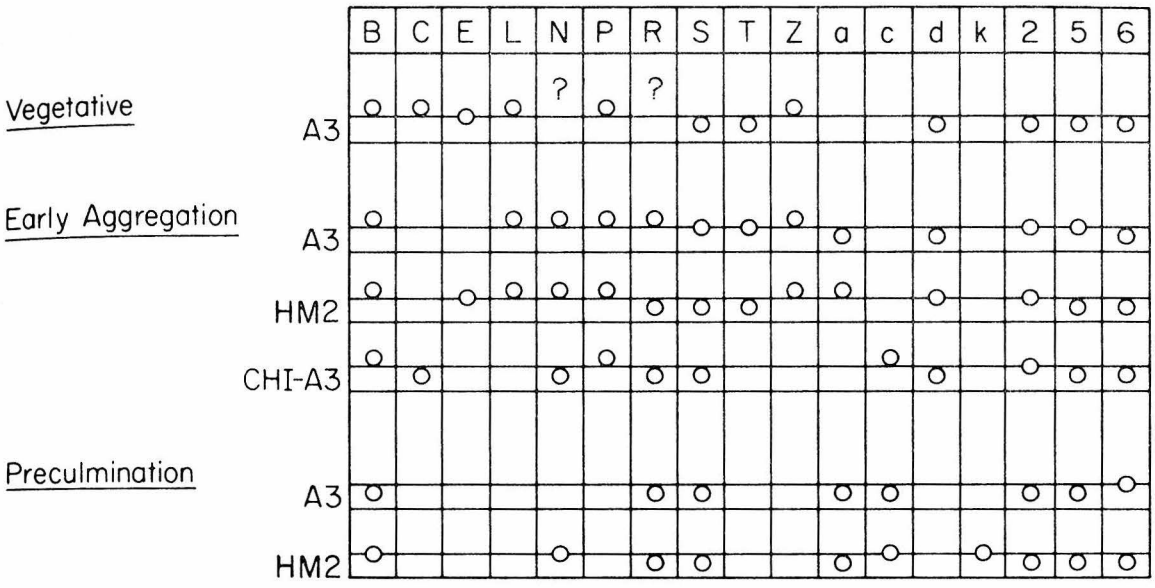
Pronase treatment of intact cells

Intact cells were pronase treated before plasma membrane purification to identify the macromolecules exposed at the exterior cell surface. We previously identified (6) 17 polypeptides present on the cell surface at some stage during development of D. discoideum. Many of these polypeptides changed in their apparent pronase sensitivity during development. Table 3 shows that there are many differences in the spectrum of sensitive polypeptides between A3 cells and cells inhibited in development.

Polypeptides such as L and Z in Fig. 3 (compare lanes 7 and 8) that are completely removed from the plasma membrane by pronase treatment of intact HM 2 cells are indicated in Table 3 as completely exposed on early aggregation HM 2 cells, and are flagged in the figures at the appropriate position. Bands that are only partially removed by pronase treatment of intact preculmination HM 2 cells such as B and c in Fig. 4 (compare lanes 2 and 3) are similarly flagged but are diagrammatically indicated in Table 3 as being partially exposed in preculmination HM 2 cells. Polypeptides that are not pronase-sensitive at a particular developmental stage or in a particular treatment are not flagged in the figure and are indicated in Table 3 as being wholly protected from proteolysis.

As described above, several pronase-sensitive polypeptides disappear from cells that have been treated with cycloheximide. In addition, five other normally pronase-sensitive polypeptides are

TABLE 3. Comparative Pronase Sensitivity of Polypeptides on Intact Cells. This table was compiled from Figures 3 and 4 as described in the text. An exposed circle represents complete pronase sensitivity, a partially exposed circle represents partial pronase sensitivity, and an unexposed circle represents insensitivity to pronase. No circle indicates the polypeptide is absent from plasma membranes from untreated cells. Variably pronase sensitive polypeptides are treated as insensitive. Polypeptides N and R are variably present in vegetative A3 plasma membranes so their sensitivity to pronase at this stage has not been determined. The data for A3 cells [a]re taken from reference 6. CHI-A3-Cycloheximide-treated A3 cells.


B C E L N P R S T Z a c d k 2 5 6
Vegetative
A3
?
?
Early Aggregation
A3
HM2
CHI-A3
Preculmination
A3
HM2

present but not sensitive to pronase when cells are incubated with cycloheximide. Two of these, including polypeptide S (6) which is discoidin (7), normally become pronase-sensitive during development but do not when cells are cycloheximide treated. Therefore, cycloheximide treatment decreases the number of exposed polypeptides by causing different species of polypeptide to disappear completely from the plasma membrane, lose their cell surface exposure, or fail to gain cell surface exposure.

Table 3 shows that when the aggregation minus mutant, HM 2, is compared to A3 cells, 5 polypeptides are found to be more pronase-sensitive in A3 cells. Four of these polypeptides, including discoidin (polypeptide S) become sensitive to pronase during development in A3 cells but do not in the mutant. In contrast, three polypeptides are sensitive to pronase in the mutant although normally not sensitive in the wild-type cells. One polypeptide becomes insensitive during development in A3 cells, but does not in the mutant.

Although we found that no glycoproteins were pronase-sensitive in intact A3 cells (6), several glycoproteins did become sensitive during inhibited development (Table 4A).

Pronase treatment of purified plasma membranes

To examine further plasma membrane topography, we pronase-treated purified plasma membranes. We assume that those macromolecules destroyed by this treatment are exposed at the plasma membrane's cytoplasmic face unless they had been shown to be exposed at the exterior cell surface by pronase treatment of intact cells (6).

TABLE 4. Pronase Sensitivity of Glycoproteins. This table compares the results previously found for pronase sensitivity of A3 plasma membrane glycoproteins (6) with those indicated in Figures 5, 6, and 9 of this paper for cells inhibited in development. Only glycoproteins whose sensitivity varies between A3 and inhibited cells or which are aberrant changes are included in this table.

S = sensitive

I = insensitive

- = absent before pronase treatment

CHI-A3 = cycloheximide treated A3 cells

Table 4

Pronase Sensitivity of Glycoproteins

Glycoprotein	Vegetative Cells	Early Aggregation			Preculmination	
	A3	A3	HM 2	CHI-A3	A3	HM 2
a) Whole cell treatment*						
M	I	I	-	S	I	I
P	I	I	S	S	-	-
Z	-	-	-	S	-	-
a	I	-	-	S	-	-
b) Plasma membrane treatment†						
C	I	I	S	S	-	S
E	I	I	S	S	-	S
G	I	I	S	S	-	S
I	I	-	-	S	-	-
K	I	I	S	I	I	I
Z	-	-	-	S	-	-
a	I	-	-	S	-	-
b	-	-	-	I	-	-
c	-	-	-	-	-	I

* All glycoproteins not listed were uniformly insensitive to pronase treatment of intact cells.

† All glycoproteins not listed were uniformly sensitive to pronase treatment of purified plasma membranes with the exceptions of N(gp) and O(gp) which were uniformly insensitive.

When development is inhibited, several glycoproteins become sensitive to pronase treatment of purified plasma membranes. A number of glycoproteins which exhibit this phenomenon are indicated in Table 4B.

Four polypeptides which are normally pronase-resistant also are sensitive in HM 2 in both early aggregation and preculmination (compare arrows in Fig. 3, lane 9 and Fig. 4, lane 1 with arrows in Fig. 3, lane 4 which is similar to Fig. 3, lane 3 in ref. 6). Two of these polypeptides are probably the polypeptide portions of H (gp) and K (gp) (6).

Therefore, inhibition of development causes a change in the exposure of several plasma membrane macromolecules. The greater efficacy of the HM 2 mutation for the disruption of membrane topography during development contrasts with the greater ability of cycloheximide to prevent changes in macromolecular composition.

DISCUSSION

In order to fully interpret the inhibition of changes in plasma membrane composition caused by the inhibition of development, it is necessary to assess the effectiveness of each treatment used to block development. As described above, HM 2 is probably blocked very early in development, but we cannot exclude the possibility that some early developmental events occur. Cycloheximide treatment is immediately effective in blocking both protein synthesis (Fig. 1) and the appearance of developmental enzymes (ref. 17, p. 112). Therefore, cycloheximide treatment is probably immediately effective in the

inhibition of development. However, the effects of cycloheximide on developing cells should not be assumed to simply result from inhibition of protein synthesis (25).

Most of the developmental changes in plasma membrane composition studied in this paper are blocked by either cycloheximide treatment or the use of the aggregation minus mutant HM 2, but all but one of the changes which were blocked by only one treatment were blocked by cycloheximide. Most (7 of 10) of the changes blocked only by cycloheximide are early developmental changes.

Although cycloheximide treatment is very effective in inhibiting developmental changes in plasma membrane composition, some normal changes still do occur in the presence of cycloheximide. As might be expected, most of these changes (9 of 10) involve decreases in the amount of components. However, this should not be taken to mean that the disappearance of D. discoideum membrane molecules is a completely passive phenomenon since the data shows that inhibition of development is able to prevent the normal disappearance of many plasma membrane macromolecules, particularly glycoproteins.

Plasma membrane glycoprotein composition is tightly linked to normal development. We previously showed (6) that 24 of 25 glycoprotein species detected with the Periodic Acid-Schiff's (PAS) method change in amount present on the plasma membrane during development. This paper shows that when development is inhibited, many of these changes do not occur.

In the course of this work, we found changes in plasma membrane composition in inhibited cells that were not previously observed in normally developing cells. Cycloheximide treatment caused the unexpected decreased or disappearance of 12 polypeptides. Cycloheximide seems to promote the selective disappearance of external polypeptides since 50% of the polypeptides which disappeared are external components whereas only 20% of all of the plasma membrane polypeptides are external (6). This may reflect a more rapid turnover of external polypeptides. This possibility has also been proposed for external polypeptides of cultured epithelial cells (26). The one developmental change that was allowed in cycloheximide treated cells but was blocked in HM 2, the loss of polypeptide E, may have been a result of this cycloheximide-induced loss of cell surface polypeptides since E was previously shown to be an external protein.

Two polypeptides appear to increase prematurely in inhibited cells. However, these changes cannot be interpreted without additional criteria for identity between these molecules and their putative normal homologs.

We also observed several polypeptides and glycoproteins in inhibited cells which were not seen previously in normally-developing wild-type cells (6). Some possible explanations for this phenomenon are: (1) These molecules are processed to other components in normally developing cells but the processing is part of the normal developmental program; (2) These molecules bind nonspecifically to the abnormal plasma membrane of inhibited cells; (3) These molecules

normally appear only transiently, but their life is extended by inhibition of development. We cannot differentiate between these possibilities at present.

Plasma membrane topography is profoundly affected by the inhibition of development. In addition to the loss of several external polypeptides mentioned above, cycloheximide treatment causes several polypeptides to lose their cell surface exposure. Several of the polypeptides which previously were observed to change their exposure on the surface during development (9), did not when development was inhibited. Therefore, it appears that changes in plasma membrane topography appear to be part of the normal program of development.

The variations in external exposure of polypeptides between normal and inhibited cells as assayed by sensitivity to pronase may be caused by one or more of several mechanisms. Sensitivity of a molecule could be modulated by shielding with other plasma membrane components. Exposure could also be controlled by interaction with subcortical networks of microtubules or microfilaments (27). Cellular ATP level has also been shown to control exposure of membrane proteins in E. coli (28). Although no glycoproteins in intact wild type cells are pronase sensitive (6), a few sensitive glycoproteins are found on cells inhibited in development. We previously found (6) that pronase treatment of purified plasma membranes destroyed about one-half the glycoprotein species. Similar treatment of plasma membranes from inhibited cells destroys a much larger number of glycoprotein species, particularly in HM 2.

The increased sensitivity of some glycoproteins to pronase treatment of cells may also be explained by the mechanisms suggested for polypeptides In addition, defective glycosylation could also cause increased pronase sensitivity. Cycloheximide treatment is known to inhibit glycosyl transferases (29) and thus could cause defective glycosylation. Glycoprotein E appears to be abnormally glycosylated in HM 2 vegetative plasma membranes (manuscript in preparation). The sensitivity of some glycoproteins to pronase treatment of isolated plasma membranes may also reflect increased exposure at the plasma membrane's cytoplasmic face.

We previously found that the lectin, discoidin (polypeptide S), is present and exposed variably (6) in vegetative wild type A3 cells. It is always exposed at early aggregation as determined by pronase sensitivity. Here we find that this developmental change in topography is blocked when development is blocked. Therefore, our results are consistent with development control of the exposure of this lectin.

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CHAPTER III

PLASMA MEMBRANE GLYCOPROTEIN COMPOSITION OF LATE AGGREGATION PHASE CELLS AND SIMILARLY INCUBATED CELLS INHIBITED IN DEVELOPMENT

SUMMARY

Plasma membrane glycoprotein composition of late aggregation A3 cells is intermediate between that of early aggregation and preculmination cells. No late aggregation specific glycoproteins were detected. Similarly incubated cells inhibited in development by mutation or cycloheximide treatment are blocked in most developmental changes. The time course of the normal changes that do occur in inhibited cells are frequently altered.

INTRODUCTION

Many plasma membrane activities appear or reach their maximal levels as cells of Dictyostelium discoideum attain full aggregation competence. These activities include the adhesion mediating contact site A antigen (1), cAMP binding sites (2), cAMP phosphodiesterase (3,4), and adenyl cyclase (5). Contact sites A and cAMP phosphodiesterase have been suggested to be glycoproteins (6,7). To obtain a more complete picture of the development changes in plasma membrane glycoprotein composition, I have extended my studies from Chapters I and II on glycoprotein composition during normal and inhibited development to the late aggregation stage of development.

METHODS

Cells

Cells used in this work were D. discoideum strains A3 and HM 2 (a spontaneous aggregation minus mutant). Cells were cultured as previously described (8).

Preparation and analysis of samples

Cells were harvested, washed, resuspended, and plated on filters as previously described (8). In experiments with cycloheximide, the one-half concentration lower pad solution (9) used to resuspend the cells and soak the filters was supplemented with 2 mg/ml cycloheximide. All incubations of cells on filters were 12 h. Cells were then harvested and plasma membranes prepared and analyzed on SDS gels for glycoprotein composition as previously described (8,10).

RESULTS AND DISCUSSION

Development of Cells

After 12 h on filters, A3 cells have collected into rounded aggregates (late aggregation stage). Similarly incubated HM 2 cells and cycloheximide treated A3 cells show no signs of morphogenesis (11, 12). They remain in a smooth lawn as when they were first put on the filters.

Plasma Membrane Glycoprotein Composition

The plasma membrane glycoprotein composition of late aggregation A3 cells (Fig. 1, lane 2) is intermediate between that previously described (8) for early aggregation and preculmination plasma membranes. This result is consistent with the developmental order of these stages. Two developmental increases, S (gp) and X (gp), and 5 decreases, G (gp), J (gp), K (gp), N (gp) and O (gp), previously found to occur by preculmination have occurred by late aggregation. On the other hand, 3 decreases, C (gp), E (gp), and P (gp), must occur between late aggregation and preculmination since they have not occurred by late aggregation. No glycoproteins were found to be present only during the late aggregation stage of development.

Few changes in plasma membrane glycoprotein composition occur in inhibited cells after the early aggregation stage of development. Of the changes that can occur, Figure 1 indicates that the decreases in N (gp), O (gp), and P (gp) in cycloheximide treated cells (lane 1) all must occur after 12 h on filters since they have not occurred by this stage. In HM 2 plasma membranes (lane 3), decreases in J (gp)

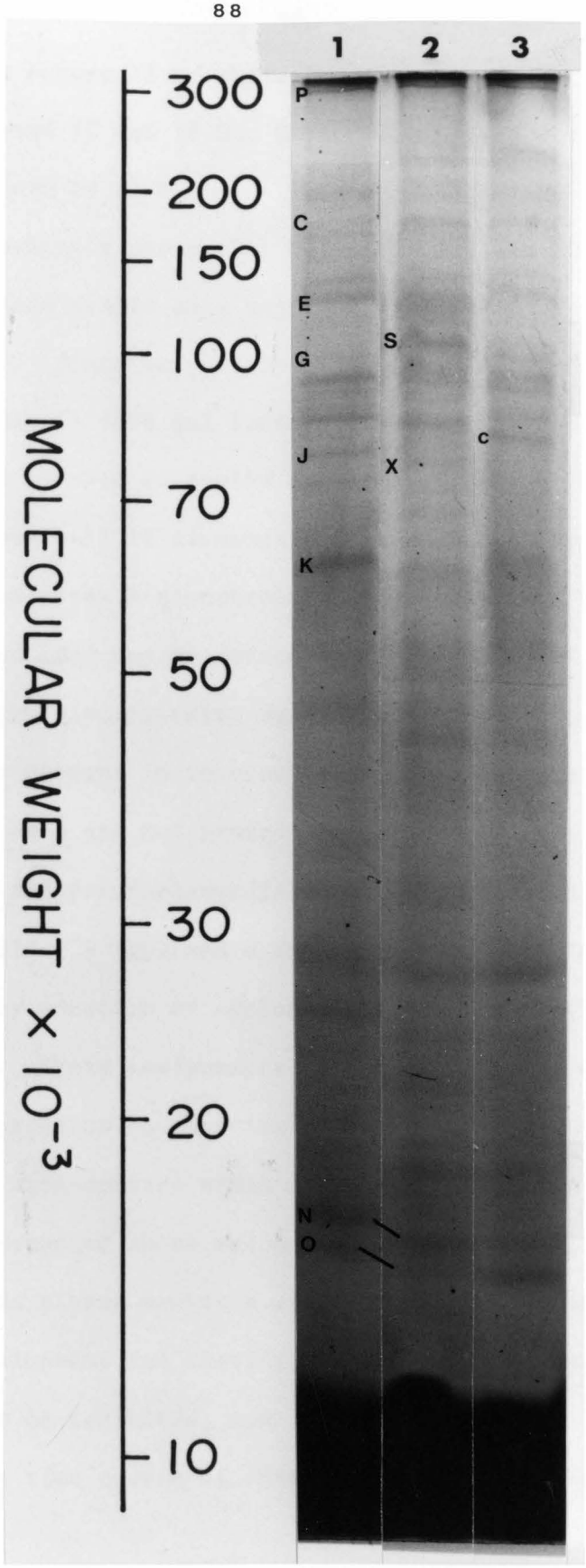
FIGURE 1. SDS gel electrophoresis of plasma membrane glycoproteins from cells incubated on filters for 12 h. Lanes from left to right are:

1) Plasma membranes from cycloheximide treated A3 cells;

2) Plasma membranes from A3 cells;

3) Plasma membranes from HM 2 cells.

Letters are placed to the left of the band they identify the first time that band appears in the figure.


1
2
3
300
200
150
100
70
50
30
20
10
MOLECULAR WEIGHT × 10⁻³
P
C
E
G
J
K
N
O
S
X
c

and N (gp) occur before 12 h, while the decrease in O (gp) and P (gp) occurs between 12 and 18 h. Glycoprotein c, observed only in HM 2 cells, appears by 12 h.

We have previously shown that we can detect 1 x 10^{14} molecules of the glycoprotein avidin as a major periodic acid-Schiff's base stained band (8). Since we put approximately 2 x 10^{8} cell equivalents of plasma membrane in each gel lane analyzed for glycoproteins, we should therefore be able to easily detect a glycoprotein present in 5 x 10^{5} copies per cell if it contained as much carbohydrate as avidin. The contact sites A glycoprotein is present in 3 x 10^{5} copies per cell (14) and thus may be detectable. S (gp) and X (gp) are the most likely of the glycoproteins we have detected to be contact sites A. They are not present in vegetative or early aggregation cells (8) when contact sites A are not present. They are present in late aggregation cells and pseudoplasmodial cells (8) when contact sites A are present (1,15). S (gp) and X (gp) are absent from cells inhibited in development by mutation or cycloheximide treatment (13) as are contact sites A. These assignments must remain purely speculative until the glycoproteins in question can be treated directly for reactivity with anti-contact sites A antiserum.

The data presented above and summarized in Table 1 substantiate the fact that the plasma membrane of D. discoideum changes in composition during development and clarify the time course of the changes. When development is inhibited, most normal changes do not occur. Furthermore, the time course of normal changes not affected by the

Table 1

Time of Occurrence of Changes in Plasma Membrane Glycoprotein Composition Previously Shown to Occur between Early Aggregation and Preculmination

Cells	Developmental Increases		Developmental Decreases	
	Before 12 h	After 12 h	Before 12 h	After 12 h
A3	S,X	---	G,J,K,N,O	C,E,P
HM 2	C	---	J,N	O,P
CHI-A3	---	---	---	N,O,P

The plasma membrane glycoprotein composition of cells incubated for 12 h on filters (Fig. 1) was compared to that previously observed (8,13) for early aggregation and preculmination plasma membranes. This comparison allowed a more precise resolution of the time at which the changes previously found to occur by preculmination actually occur (i.e., before or after 12 h incubation on filters).

CHI-A3 = Cycloheximide treated A3 cells.

inhibition of development is differentially altered depending on the means of inhibition. For example, glycoproteins N, O, and P all decrease during incubation whether or not development is inhibited. In A3 cells, N (gp) and O (gp) decrease before 12 h and P (gp) decreases after 12 h. However, in HM 2 cells, only N (gp) decreases before 12 h, while in cycloheximide treated A3 cells all 3 decreases occur after 12 h. This shows, in agreement with previous results, that cycloheximide treatment is more effective than mutation in blocking changes in plasma membrane composition.

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CHAPTER IV

DEFECTIVE GLYCOPROTEINS IN THE PLASMA MEMBRANE OF AN AGGREGATION MINUS MUTANT OF *DICTYOSTELIUM DISCOIDEUM* WITH ABNORMAL CELLULAR INTERACTIONS

DEFECTIVE GLYCOPROTEINS IN THE PLASMA MEMBRANE OF AN AGGREGATION MINUS MUTANT OF *DICTYOSTELIUM DISCOIDEUM* WITH ABNORMAL CELLULAR INTERACTIONS[1]

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Summary

Experiments involving the coincubation of wild type (A3) cells of D. discoideum and a spontaneous aggregation-minus mutant (HM 2) suggested that the mutant was defective in cellular interactions. The inhibition of A3 development by HM 2 cells and the differentiation of a small fraction of HM 2 cells allowed by A3 cells depend on cell contact. Therefore, we compared cell surface molecules in vegetative A3 and HM 2 cells by a variety of techniques to determine if defects in HM 2 could be found prior to the inhibition of development in vegetative amoebae. Antigenic defects and/or differences in binding of concanavalin A were localized to three plasma membrane macromolecules using glutaraldehyde-fixed SDS gels of plasma membranes. Two periodic acid-Schiff positive glycoprotein, and one glycolipid also differed in HM 2. Three glycoproteins had an increased sensitivity to pronase in isolated plasma membranes suggesting an alteration in their topography. Glycoprotein E, the major glycoprotein of vegetative plasma membranes is abnormal in topography, as a concanavalin A receptor, and is antigenically abnormal. No differences were found in polypeptide composition and topography or in phospholipid composition between A3 and HM 2 plasma membranes. Most or all of the aberrant molecules contain carbohydrate.

INTRODUCTION

Developmental mutants have frequently been used to study the biochemical parameters associated with development in Dictyostelium discoideum. When development is blocked many normal changes do not occur including the accumulation of stage specific enzymes (1), the appearance of cAMP receptor and phosphodiesterase (2,3), and of contact sites A (4). Thirteen normal developmental alterations in the spectrum of macromolecules and five developmental changes in polypeptide topographical location in the plasma membrane of D. discoideum are blocked in a developmental mutant.[2] Although several biochemical failures are associated with developmental mutants, many of these effects could be secondary results of the inhibition of the developmental program.

Developmental mutants have been shown by Sussman and collaborators to participate in a variety of synergistic or antagonistic interactions when coincubated in pairs or with wild type cells under conditions suitable for development. Two mutants were found to inhibit the development of wild type cells during coincubation (5). On the other hand, pairs of mutants, or a mutant strain and wild type cells can synergize to allow development of cells that would not have otherwise differentiated (6-8). Sussman, Raper, and their coworkers indicated the importance of the cell surface in these phenomena by their demonstration that when the different cell populations are separated by thin membranes easily permeable to diffusible molecules, the interactions are frequently blocked (5-8). One interpretation of these data is that these phenomena are mediated by cell surface molecules that must contact each other for an interaction to take place (40).

Cell surface glycoproteins have many intriguing roles in the development of Dictyostelium. The cohesion-mediation contact site A antigen detected by Beug et al. (4) is a glycoprotein. Three plasma membrane enzymes, including cAMP phosphodiesterase, have recently been suggested to be glycoproteins and to change in their carbohydrate structure during development (9). The binding of the lectin, Con A[3], to cell surface glycoproteins can stimulate or inhibit development depending on the length of the incubation (10-12). It also prematurely induces cAMP phosphodiesterase activity (10,11). The inhibitory effect of pseudoplasmodial plasma membranes on the resumption of normal morphogenesis by dissociated pseudoplasmodial cells can be destroyed by periodate oxidation, but not by extensive proteolysis suggesting that the exposed molecules mediating this process are carbohydrates.[4] Major changes occur in plasma membrane glycoprotein composition during development (13-16).

Immunological studies have been useful in the understanding of the role of the cell surface in the development of D. discoideum. Contact sites A, which appear during aggregation and mediate cellular cohesion, were defined immunologically (4). Sonneborn et al. have also defined antigens associated with particulate cellular components found only on aggregrating cells (17,18). An antigen exists that is found specifically on spore cells (19). Gregg and Trygstad found that antisera against wild type amoebae fails to agglutinate some aggregation minus mutants and vice versa (20). Therefore, it is clear that antigens on the cell surface of D. discoideum change during development and that some mutants unable to aggregate may have defective cell surfaces. However, the molecular nature of developmentally controlled antigens has not previously been determined.

In order to find a defect or defects in an aggregation minus mutant that was unlikely to be a secondary effect of the inhibition of development, and therefore more likely to be a direct result of the primary genetic lesion, we examined the plasma membrane of vegetative cells of such a mutant by a variety of independent techniques. A spontaneous mutant was used to minimize the possibility of there being mutations present irrelevant to the developmental defect. We found six differences between the mutant and wild type plasma membrane, at least five of which are carbohydrate containing molecules.

EXPERIMENTAL PROCEDURE

Cells

Cells used are D. discoideum strain A3, and a spontaneous aggregation minus mutant, HM 2, isolated from A3 cells by D.M.

Development of cells in suspension and assay of contact site A formation

To test for the ability of cells to form contact sites A (4), 5 ml of A3 or HM 2 cells in suspension at 1 x 10^7 cells/ml in aggregation buffer (21) were rotated at 20-24 revolutions per min in a 25 x 200 mm screw-top culture tube about the tube's long axis in a device built according to Gerisch's description (22).

After 12 hours at 22°, aliquots of cell suspension were photographed in the presence or absence of 10 mM EDTA. If cells remained aggregated in the presence of EDTA, they were assumed to have formed contact sites A (4).

Development of cells on filters

A3 and/or HM 2 cells were washed and resuspended in lower pad solution (23). The indicated numbers of A3 cells, HM 2 cells, or a com-

bination of the two were uniformally spread on a 47 mm diameter millipore filter on a pad saturated with lower pad solution and incubated for development at 22°.

In experiments where cells were incubated on opposite sides of millipore filters from each other, the appropriate number of A3 or HM 2 cells were spread onto each filter and the filters were clamped together back to back with a stainless steel ring and suspended horizontally 5 mm above a lower pad solution soaked pad.

Triton X-100 treatment

Spores were treated with 0.2% Triton X-100, and suitable dilutions were mixed with bacteria, and plated on SM agar (24). Fewer than 1 in 10^7 amoebae survive this treatment although it has no effect on the viability of spores.

Plasma membrane preparation and SDS gel electrophoresis

Cells were grown, plasma membranes prepared (25) and analytical SDS gels were run and stained as previously described (13). The Coomassie blue stained gel in Fig. 10 contains 50 µg protein per lane and the PAS stained gel in Fig. 8 contains 200 µg protein per lane as estimated by the Lowry method (26). Pronase treatment of intact cells and isolated plasma membrane was also as previously described (13).

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed as described by O'Farrell (27) and Ames and Nikaido (28) with the following slight modifications.

Plasma membrane samples are solubilized using SDS. Two mg plasma membrane protein is pelleted by centrifugation at 48,000 g for 15 min in

an SS34 rotor and the supernatant removed. 0.1 ml isoelectric focusing dissociation buffer (0.05 M Tris pH 6.8, 2% SDS, 0.5 mM $MgCl_2$, 10% (v/v) 2-mercaptoethanol) is added, and the sample is incubated 30 min at 70°. Then 0.2 ml of a solution 9% (v/v) in Triton X-100 and 2% in ampholine is added followed by 100 mg urea. 100 µl of the supernatant containing 450 µg protein is used per isoelectric focusing gel.

Isoelectric focusing gels were calibrated by incubating 5 mm slices of a gel that had been run with no sample in 2 ml of a degassed solution of 9.12 M urea. The pH of the solution was measured after 5 hours.

Scanning electron microscopy

Cells were fixed with glutaraldehyde, postfixed with OsO_4 dehydrated with an ethanol-series, and critical-pointed dried. Before viewing the samples were shadowed with gold.

Lectin and antibody labeling of gels

SDS slab gels were run (13) and then cut into 0.7 cm wide gel strips containing 120 µg of plasma membrane protein. The strips were then fixed as described in reference 29. For lectin binding, gel strips were incubated for 3 days in 8 ml of infusion solution (29) per gel strip plus 0.25 mg FITC-lectin per gel strip. They were then washed for 2 days in phosphate buffered saline (29) with 2 changes of solution and photographed over a short wavelength (predominantly 254 nm) UV light box through a Wratten type 65 filter (14). For antibody binding, gel strips were incubated for 3 days with 5 ml rabbit serum plus 3 ml 2.67 x infusion solution per gel strip. The gels strips were then washed as above to remove unbound antibody and incubated for 3 days with 8 ml infusion

solution plus 5 mg FITC-goat anti-rabbit immunoglobulin per gel strip. Finally the gels were washed and photographed as above.

Gel scanning

Photographic negatives of gels were scanned on a Syntex AD-1 Autodensitometer.

Preparation, titering and adsorption of sera

Rabbits were inoculated with A3 vegetative plasma membrane (10 mg protein content) suspended 1:1 (v/v) with complete Freund's adjuvant in a final volume of about 2 ml. Equal aliquots were injected intramuscularly, intradermally, and subcutaneously. Similar inoculations were given four weeks later and weekly for 3 weeks thereafter. Starting at week 5, the rabbits were bled about 25 ml weekly from the ear. After 3 hours at room temperature the serum was separated from the blood clot by centrifugation for 10 min at 4200 g.

To titer sera, vegetative A3 cells were washed and resuspended in 0.15 N NaCl* plus 2 mM EDTA (pH 6.0) at 4×10^7 cells per ml. 0.1 ml of cells were mixed with 0.1 ml of a range of serum dilutions and the highest dilution to give complete agglutination was determined. Typically titers were 64 for immune serum and 2 for preimmune serum.

Adsorption of a titer 64 serum with 3.2×10^8 A3 or HM 2 cells per ml for 30 min completely removes all agglutinating activity. However, when pooled immune sera from 2 rabbits (average titer 64) was adsorbed with a sufficient number of A3 cells to completely adsorb 128 units of agglutinating titer, detectable antibody binding to fixed gels of A3 vegetative plasma membranes remained (data not presented). Repeating this adsorption a total of 3 times removed almost all antibody

binding to fixed gels. Therefore, A3 and HM 2 adsorption of anti-A3 vegetative plasma membrane antiserum with cells of A3 or HM 2 was routinely done by 3 rounds of adsorption, each with 6.4×10^8 cells per ml of serum.

Preparation of lipids and thin layer chromatography

Total cell or plasma membrane lipids were prepared as described in reference 30 page 227, and partitioned once as described. The upper phase was dried, dialyzed 24 hours against H_2O, dried, resuspended in chloroform-methanol (2:1, v/v), and insoluble material was removed by centrifugation. The lower phase was dried and resuspended in chloroform-methanol (2:1, v/v). The samples were analyzed by thin layer chromatography on precoated 0.25 mm silica gel plates (EM Laboratories, Darmstadt). Plates were developed with chloroform-methanol-H_2O (60:35:8, v/v/v), dried, and stained for carbohydrate with the orcinol reagent (30, p. 226) or for phospholipids (31).

Gas-liquid chromatography

A3 and HM 2 vegetative plasma membranes were resuspended at about 1 mg/ml membrane protein in 0.03 M ammonium bicarbonate, pH 7.9. Ten µl samples were dried, hydrolyzed by methanolysis, derivatized with trifluoroacetic acid, and analyzed for carbohydrate by gas-liquid chromatography.[5]

RESULTS

Development of the mutant, HM 2

The spontaneous mutant, HM 2, fails to develop normally on plates of bacteria, in suspension, or on filters resting on buffer saturated pads. When wild type, A3, cells are gently rotated for at least 6 hours

in aggregation buffer (21), large, tight aggregates are formed (Fig. 1A) that are resistant to EDTA (Fig. 1B). Similarly incubated HM 2 cells from only smaller, loose aggregates (Fig. 1C) that are sensitive to EDTA treatment (Fig. 1D) even when incubated for 12 hours.

A3 cells incubated on filters for 12 hours are in large aggregates (Fig. 2A). On the other hand, HM 2 cells are in small disperse clumps (Fig. 2B). Higher magnifications indicate that individual A3 (Fig. 2C) and HM 2 (Fig. 2D) cells are relatively similar in appearance. The only difference is the globular protrusions found on the surface of A3 cells (Fig. 2C). The surface morphology of A3 cells entering aggregates is similar to that of HM 2 cells.

Experiments in which A3 and HM 2 cells are mixed suggest that a biochemical defect in HM 2 is expressed at the cell surface. When HM 2 cells are coincubated with A3 cells, the development of the A3 cells is inhibited. When A3 cells were plated at the standard concentration, 4.7×10^7 spores developed in Experiment 1A (Table I). Similarly incubated HM 2 cells formed no spores (Experiment 1G). As might be expected, replacement of 50% of the A3 cells with HM 2 cells resulted in about a 50% decrease in number of spores formed (Experiment 1D). However, as the percentage of HM 2 cells were further increased, the number of spores formed fell drastically. Inclusion of 75% (Experiment 1E) or 87.5% HM 2 (Experiment 1F) cells decreased the number of spores found respectively to 0.78% and 0.04% of the number formed by a pure A3 culture. Control experiments with 25% (Experiment 1B) or 12.5% (Experiment 1C) of the standard number of A3 cells incubated alone indicated that, for the most part, the decrease in spores formed by coincubation A3 and HM 2 cells is

FIGURE LEGENDS

Fig. 1. Assay of contact sites A formation by developing cells. A3 or HM 2 cells at a concentration of 1 x 10^7 cells/ml were rotated for 12 hours as described in the Methods and photographed in the presence or absence of 10 mM EDTA.

A. A3 cells without EDTA;

B. A3 cells plus EDTA;

C. HM 2 cells without EDTA;

D. HM 2 cells plus EDTA.

All magnifications are 512X.

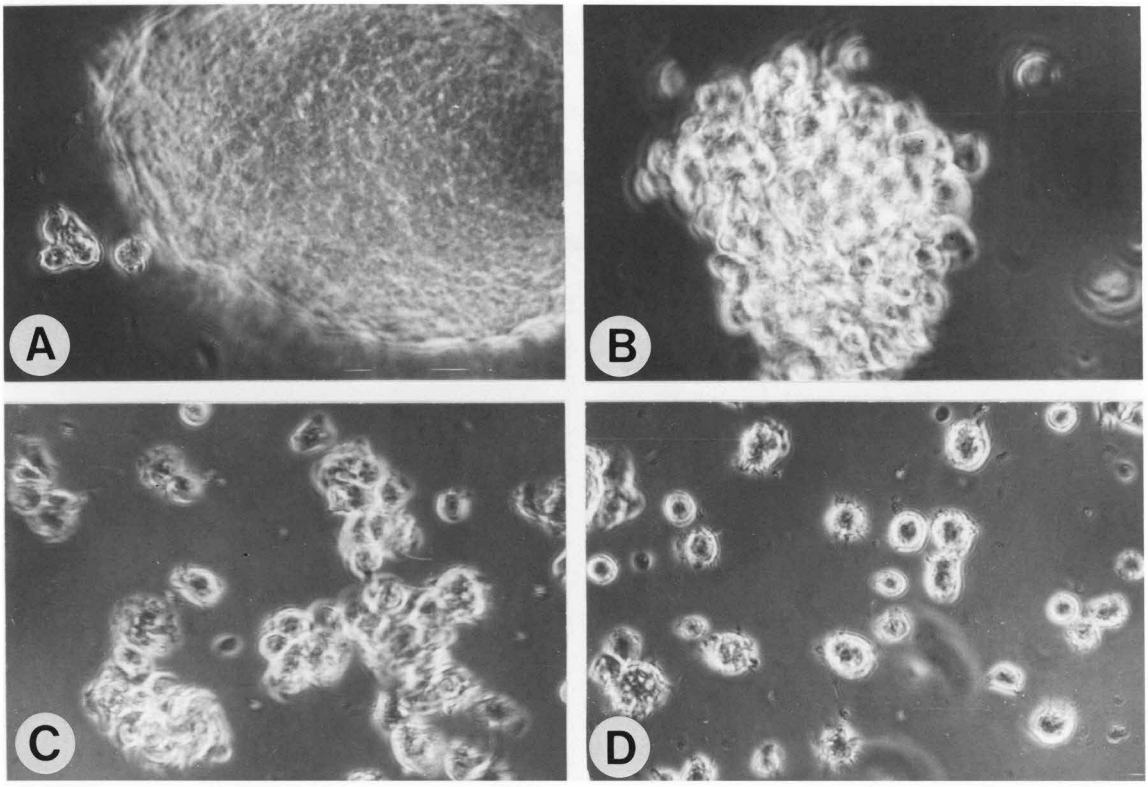

A
B
C
D

Fig. 2. Comparative scanning electron microscopy of A3 and HM 2 cells. A3 and HM 2 cells were incubated on filters for 12 hours and prepared for scanning electron microscopy as described in the Methods.

A. A3 cells (880X magnification);

B. HM 2 cells (320X magnification);

C. A3 cells (3000X magnification), globular protrusions (arrows) and the edge of the aggregate (agg) are indicated;

D. HM 2 cells (3600X magnification).

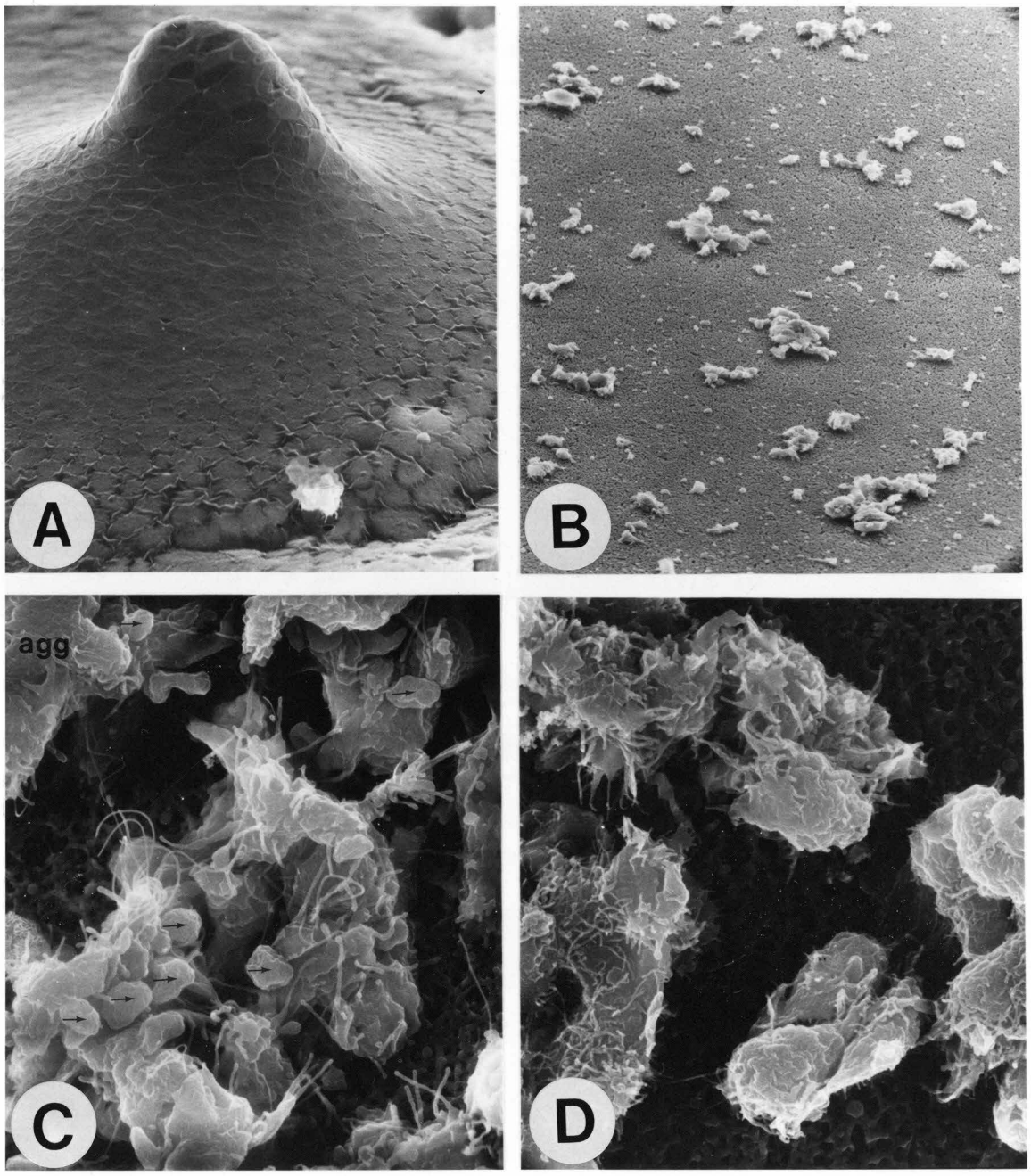

A
B
agg
C
D

Table I

Synergistic and antagonistic developmental interactions between A3 and HM 2 cells

Experiment 1

Cells		Spores/filter	% Experiment 1A
A 3/filter	HM 2/filter		
A) 4×10^7	-	4.7×10^7	100
B) 1×10^7	-	7.7×10^6	16
C) 5×10^6	-	2.4×10^6	5.1
D) 2×10^7	2×10^7	2.7×10^7	57
E) 1×10^7	3×10^7	3.7×10^5	0.78
F) 5×10^6	3.5×10^7	1.7×10^4	0.04
G) -	4×10^7	0	0
H) 1×10^7	(3×10^7 transfilter)	3.6×10^6	7.7
I) 1×10^7	(3×10^7 A3 cells transfilter)	3.3×10^6	7.0

Table I (Continued)

Experiment 2

Sorocarp	Aggregation minus plaques	
	Untreated	+ 0.2% Triton X-100
A	2/578	2/514
B	3/245	1/102
C	2/132	1/107
D	2/107	0/74
E	0/203	2/134
F	1/182	0/64
G	0/163	2/110
H-L	0/535	0/403
Total	10/2209	8/1546
Frequency aggregation minus plaques	0.45%	0.51%

In Experiment 1, the indicated number of A3 cells, HM 2 cells, or a combination of the two were incubated together or transfilter from each other as described in the Methods. The fact that the number of spores recovered under optimal conditions is greater than the original number of cells plated is consistent with previous results (32) indicating increase in cell number during development.

In Experiment 2, the spores from 12 sorocarps from experiment 1E were harvested and suitable dilutions incubated with bacteria on SM agar and the development of the plaques formed were scored. Some spores were treated with 0.2% Triton X-100 before plating to destroy any contaminating amoebae.

due to the presence of HM 2 cells and not the lower density of A3 cells present. Coincubation of A3 and HM 2 reduce both the number and size of sorocarps which are produced and slows their development (data not shown).

The inhibition of development by HM 2 cells is reversed if the A3 and HM 2 cells are separated by millipore filters. When A3 and HM 2 cells in a ratio of 1:3 are incubated so that each strain is separated by millipore filters, 10 times as many spores form as when a similar ratio of A3 and HM 2 cells are coincubated (compare Experiment 1E with 1H in Table I). A slight reduction in the number of spores formed is still observed as compared to an equivalent concentration of A3 cells (Experiment 1B), but this difference may be a result of the placement of the filters in this experiment since the number of spores formed was similarly reduced when A3 cells were plated transfilter to A3 cells (Experiment 2). Therefore, the inhibitory effect of HM 2 cell on A3 development appears to depend on cell contact.

Coincubation with A3 cells results in the development of a small fraction of HM 2 cells. Twelve individual sorocarps were picked from Experiment 1E (A3 and HM 2 cells in a ratio of 1:3), plated with bacteria, and the resultant plaques scored for the HM 2 (aggregation minus) phenotype. As indicated in Table I, Experiment 2, about 0.5% of the plaques formed were aggregation minus, suggesting that HM 2 was rescued in its ability to develop into spores but was selected against in the ratio of 150:1. Two control experiments substantiate the suggestion that the aggregation minus plaques were due to HM 2 spores which are produced by association with A3. No aggregation minus plaques were observed when pure A3 sorocarps were plated with bacteria. Treatment of spores with

Triton X-100 to destroy any HM 2 amoebae which might have accidentally contaminated the spores did not decrease the frequency of aggregation minus plaques (Table I, Experiment 2). No spores were found when pure HM 2 cultures or HM 2 cultures transfilter from A3 culture were incubated under conditions which allow development of A3. Therefore, the rescue of HM 2 also appears to depend on cell contact.

Plasma membrane antigens

To detect defective cell surface molecules in HM 2, we reacted antisera made against A3 plasma membrane with A3 and HM 2 vegetative plasma membrane macromolecules that had been resolved on SDS polyacrylamide gels and fixed in the gels with glutaraldehyde (29). In some experiments, this antiserum was adsorbed with A3 or HM 2 cells before use.

Two plasma membrane macromolecules bear antigens that are qualitatively and/or quantitatively different in A3 and HM 2. One of these macromolecules appears to be glycoprotein E (13), since purified glycoprotein E carries this antigenic difference.[6] Antigens associated with glycoprotein E will be referred to as E (Ag). E (Ag) from HM 2 plasma membranes (Fig. 3B, Fig. 4B) binds at least twice as much unadsorbed antibody as does E (Ag) from A3 plasma membranes (Fig. 3A, Fig 4A). However, HM 2 adsorption of the antiserum decreases E (Ag) antibody binding to an indetectable level in HM 2 plasma membranes (Fig. 5B, Fig. 6B), but has no effect on E (Ag) antibody binding to E (gp) from A3 plasma membranes (Fig. 5A, Fig. 6A). This differential effect of HM 2 adsorption clearly indicates that the E (Ag) of HM 2 plasma membranes is exposed at the surface of HM 2 cells but that the E (Ag) of A3 plasma

Fig. 3. Antigenic comparison of mutant and wild-type plasma membranes. Plasma membrane macromolecules were separated by SDS gel electrophoresis, fixed in the gels, and reacted with either preimmune rabbit serum or immune serum prepared against vegetative A3 plasma membranes. The antigens were then detected with fluorescent goat anti-rabbit antibodies. The details of this technique are presented in the Methods.

A. A3 plasma membrane macromolecules reacted with immune serum;

B. HM 2 plasma membrane macromolecules reacted with immune serum;

C. A3 plasma membrane macromolecules reacted with preimmune serum.

The 2 major discrete antigenic differences between A3 and HM 2 plasma membrane are indicated as E(Ag) because this antigen comigrates with E(gp) (13) and 150 (Ag) because the molecular weight of the antigen is 150,000. Also note that the broad continuous band of antigen seen in both A3 and HM 2 plasma membranes has a smaller migration in HM 2.

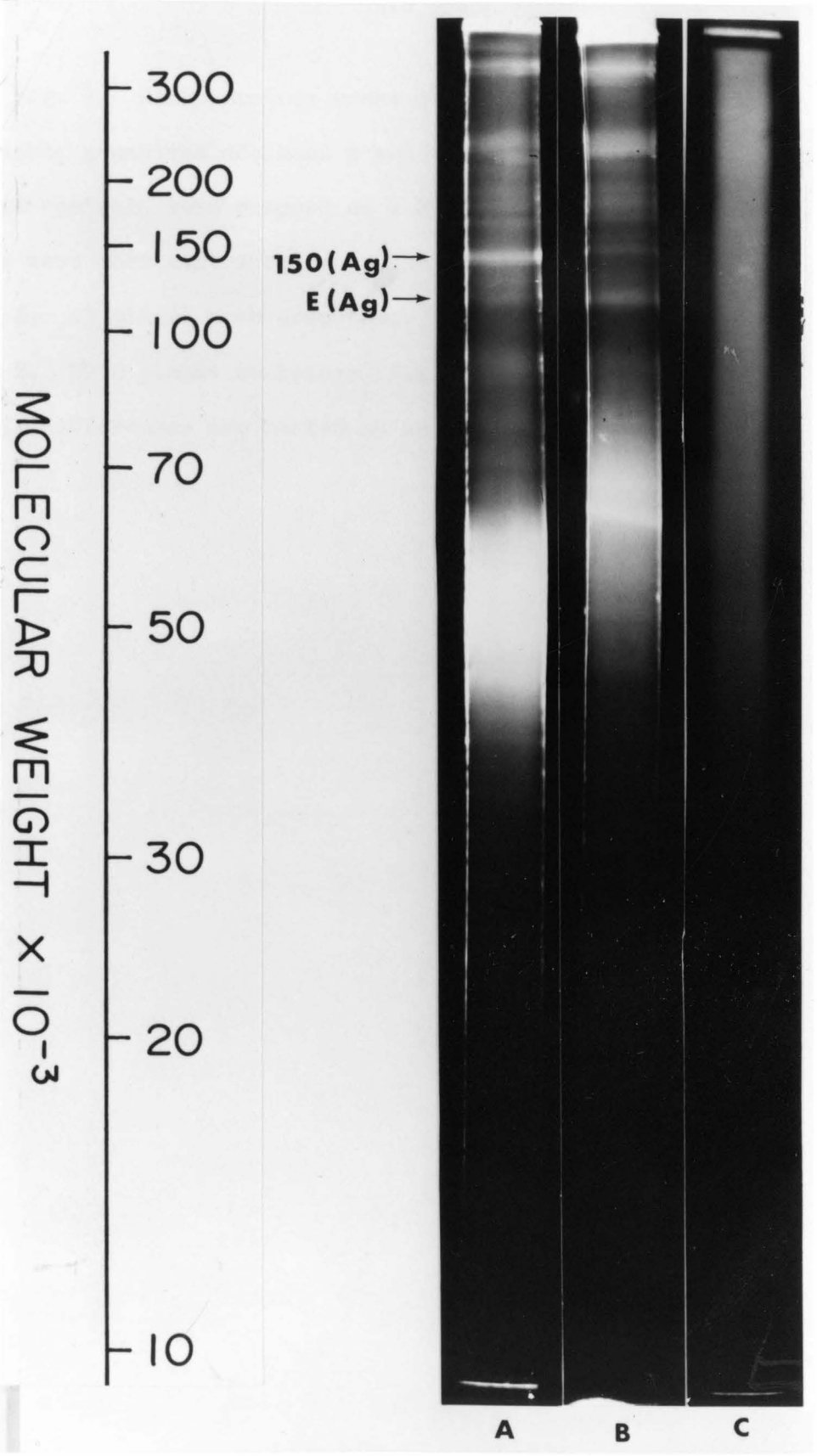

300
200
150
100
70
50
30
20
10
MOLECULAR WEIGHT × 10⁻³
150(Ag)
E(Ag)
A
B
C

Fig. 4. Densitometric scans of lanes A and B from Fig. 3. Photographic negatives of lanes A and B from Fig. 3 and the appropriate preimmune controls were scanned on a Syntex AD-1 Autodensitometer. The controls were then subtracted from the experimentals.

A. A3 plasma membranes (Fig. 3A);

B. HM 2 plasma membranes (Fig. 3B).

Antigenic differences are marked as in Fig. 3.

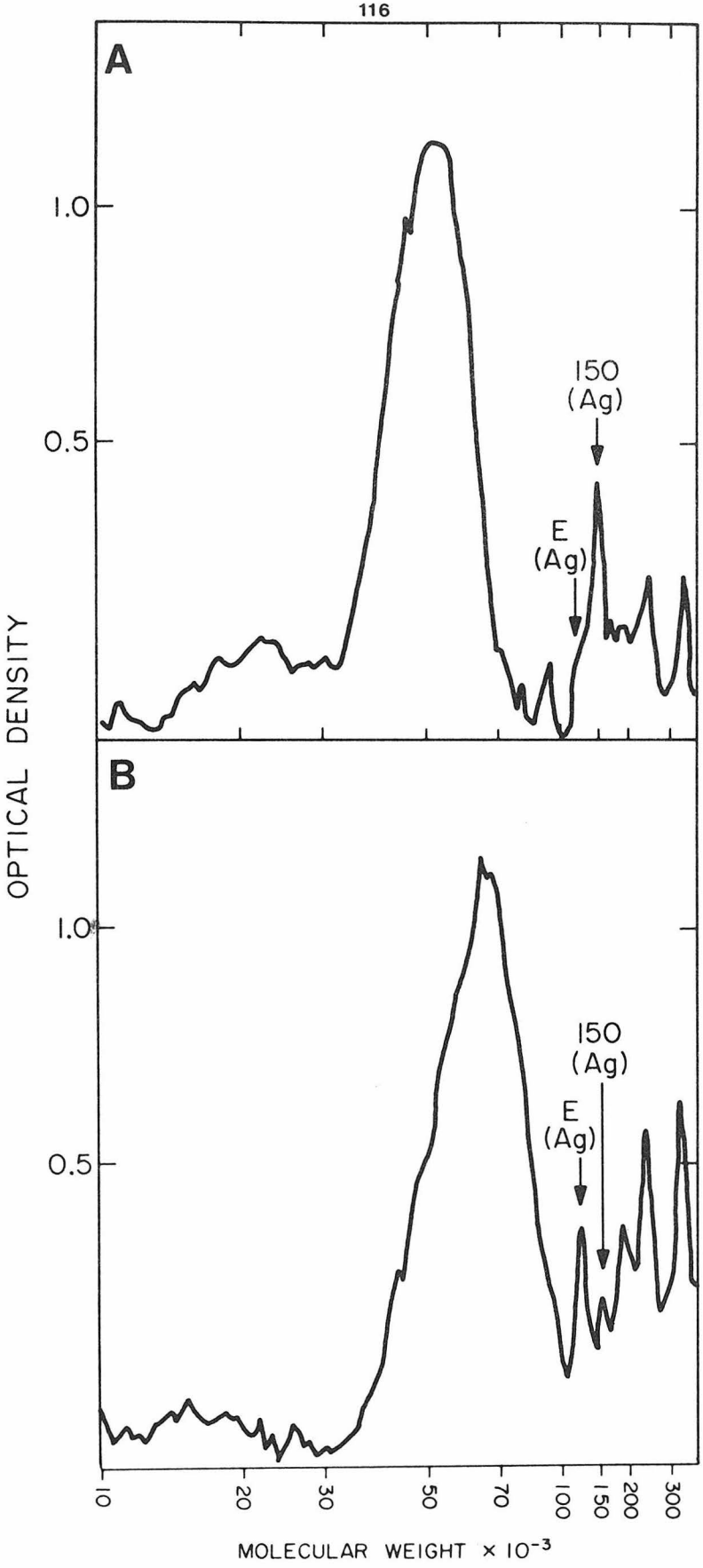

A
B
1.0
0.5
150
(Ag)
E
(Ag)
OPTICAL DENSITY
10
20
30
50
70
100
150
200
300
MOLECULAR WEIGHT × 10⁻³

Fig. 5. Identification of antigenic differences between A3 and HM 2 plasma membranes. Antiserum against vegetative A3 plasma membranes was adsorbed with either A3 or HM 2 cells and reacted with A3 or HM 2 plasma membranes after SDS gel electrophoresis as described in the Methods.

A. HM 2 adsorbed serum reacted with A3 plasma membrane macromolecules;

B. HM 2 adsorbed serum reacted with HM 2 plasma membrane macromolecules;

C. A3 adsorbed serum reacted with A3 plasma membrane macromolecules; and

D. A3 adsorbed serum reacted with HM 2 plasma membrane macromolecules. The position of E(Ag) and 150 (Ag) are indicated.

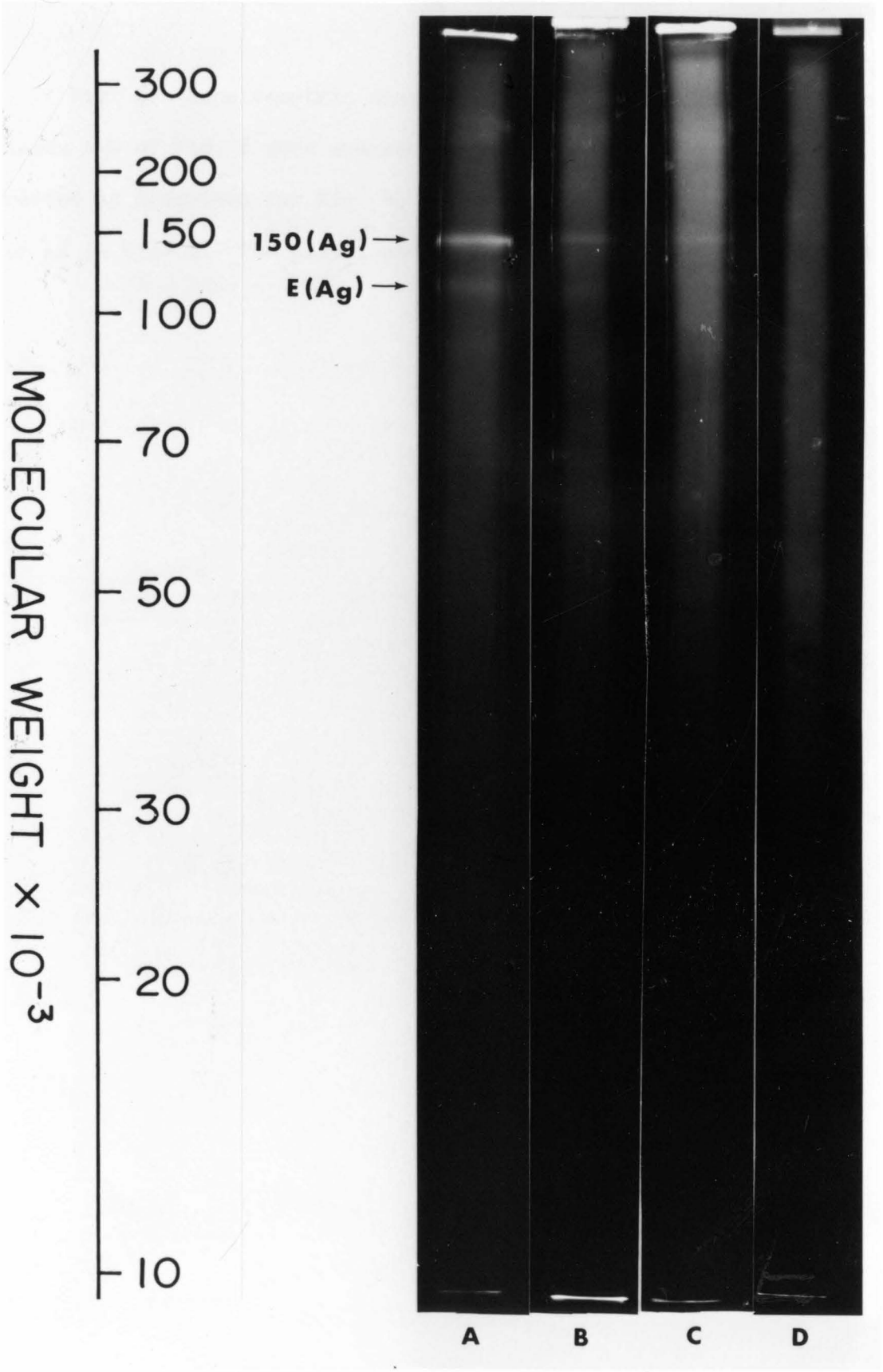
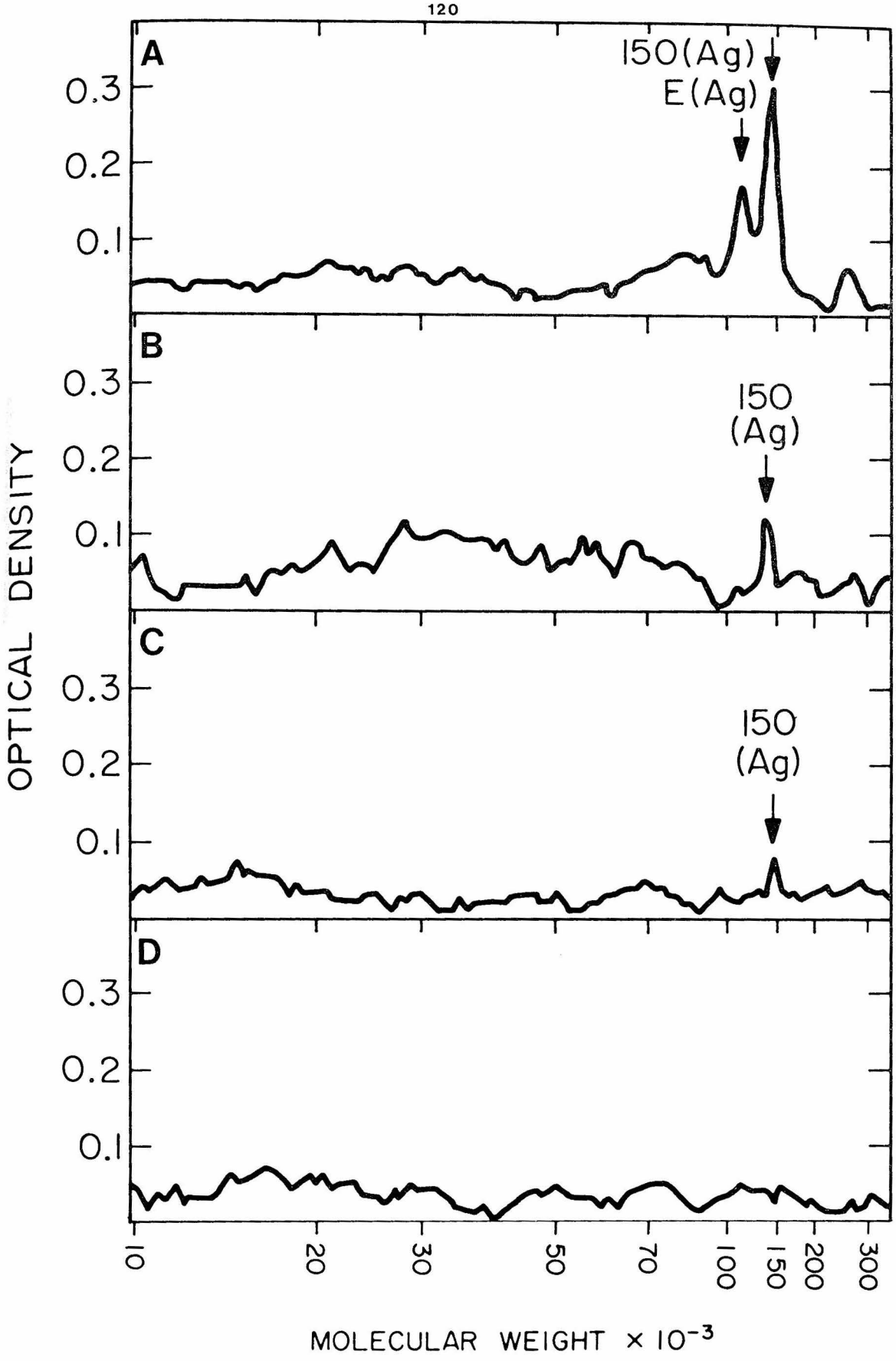

300
200
150
100
70
50
30
20
10
MOLECULAR WEIGHT × 10⁻³
150(Ag)
E(Ag)
A
B
C
D

Fig. 6. Densitometric scans of Fig. 5. Photographic negatives of lanes A-D of Fig. 6 were scanned on a Syntex AD-1 Autodensitometer, corrected as described for Fig. 4, and are presented here in the same order as in Fig. 5. The positions of E(Ag) and 150 (Ag) are indicated.


A
150(Ag)
E(Ag)
B
150
(Ag)
C
150
(Ag)
D
0.3
0.2
0.1
OPTICAL DENSITY
10
20
30
50
70
100
150
200
300
MOLECULAR WEIGHT × 10⁻³

membranes differs from the HM 2 E (Ag) so that its antibody binding is not affected by HM 2 adsorption. A3 adsorption of antiserum removes the antibodies that bind both to E (Ag) from A3 (Fig. 5C, Fig. 6C), and HM 2 cells (Fig. 5D, Fig. 6D). Therefore, both the A3 and HM 2 forms of E (Ag) are present on the A3 cell surface.

Another antigenic difference between A3 and HM 2 is associated with a macromolecule of molecular weight 150,000, referred to as 150(Ag). The 150(Ag) in A3 plasma membranes (Fig. 3A, Fig. 4A) binds more antibody from unadsorbed serum against A3 plasma membrane than does the 150(Ag) in HM 2 plasma membranes (Fig. 3B, Fig. 4B). The data using adsorbed sera are also consistent with the possibility that the only difference in 150(Ag) between A3 and HM 2 is quantitative (more in A3) although a qualitative difference cannot be ruled out. A3 adsorption reduces 150(Ag) antibody binding at least 80% in both A3 (Fig. 5C, Fig. 6C) and HM 2 (Fig. 5D, Fig. 6D) plasma membranes. On the other hand, HM 2 adsorption reduces 150(Ag) antibody binding no more than 30% to 150(Ag) from either A3 (Fig. 5A, Fig. 6A) or HM 2 (Fig. 5B, Fig. 6B) plasma membranes. The molecule bearing 150(Ag) and glycoprotein D (13) have similar molecular weights. However, there is no other evidence for their identity.

Several other antigens were detectable by unadsorbed serum in both A3 (Fig. 3A) and HM 2 (Fig. 3B) plasma membranes. However, antibodies were uniformly adsorbable by either A3 or HM 2 cells (Fig. 5). Unadsorbed serum indicated one further difference between A3 and HM 2 plasma membranes. A broad, continuous band of antibody binding was observed at a lesser migration in HM 2 (Fig. 3B, Fig. 4B) than in A3

(Fig. 3A, Fig. 4A) plasma membranes. The nature of this material is not known at present. No antigens were recognized by preimmune serum (Fig. 3C).

Lectin binding of plasma membrane glycoproteins

Since E(Ag) comigrates with glycoprotein E (13) and since 150(Ag) comigrates with glycoprotein D (13), we wished to consider the possibility that HM 2 has defective glycoproteins. Therefore, we analyzed A3 and HM 2 vegetative plasma membranes for their total carbohydrate content and for lectin binding to glycoproteins resolved on SDS gels.

The total carbohydrate compositions of A3 and HM 2 plasma membranes are very similar, with the exception of fucose (Table II). Although, there appears to be about twice as much fucose in HM 2 plasma membranes, interpretation of this point must remain tentative since the difference is not statistically significant. The major carbohydrates found in Dictyostelium plasma membranes are fucose, mannose, glucose, and glucosamine. Galactose, galactosamine and sialic acids are not detectible. In terms of carbohydrates present, these results are generally in accordance with those of Gilkes and Weeks (33). However, we detect a different ratio of carbohydrates. In particular, we find 50% more glucosamine per mg protein. In addition attempts to measure galactosamine in the plasma membrane have not been previously reported.

West and McMahon (14) have developed a method to routinely monitor the glycoprotein composition of plasma membranes by the binding of FITC-lectins to pre-fixed polyacrylamide gels of glycoproteins. Differences in lectin binding between A3 and HM 2 plasma membranes were

Table II

Plasma membrane carbohydrate composition

(μmole carbohydrate/mg protein)

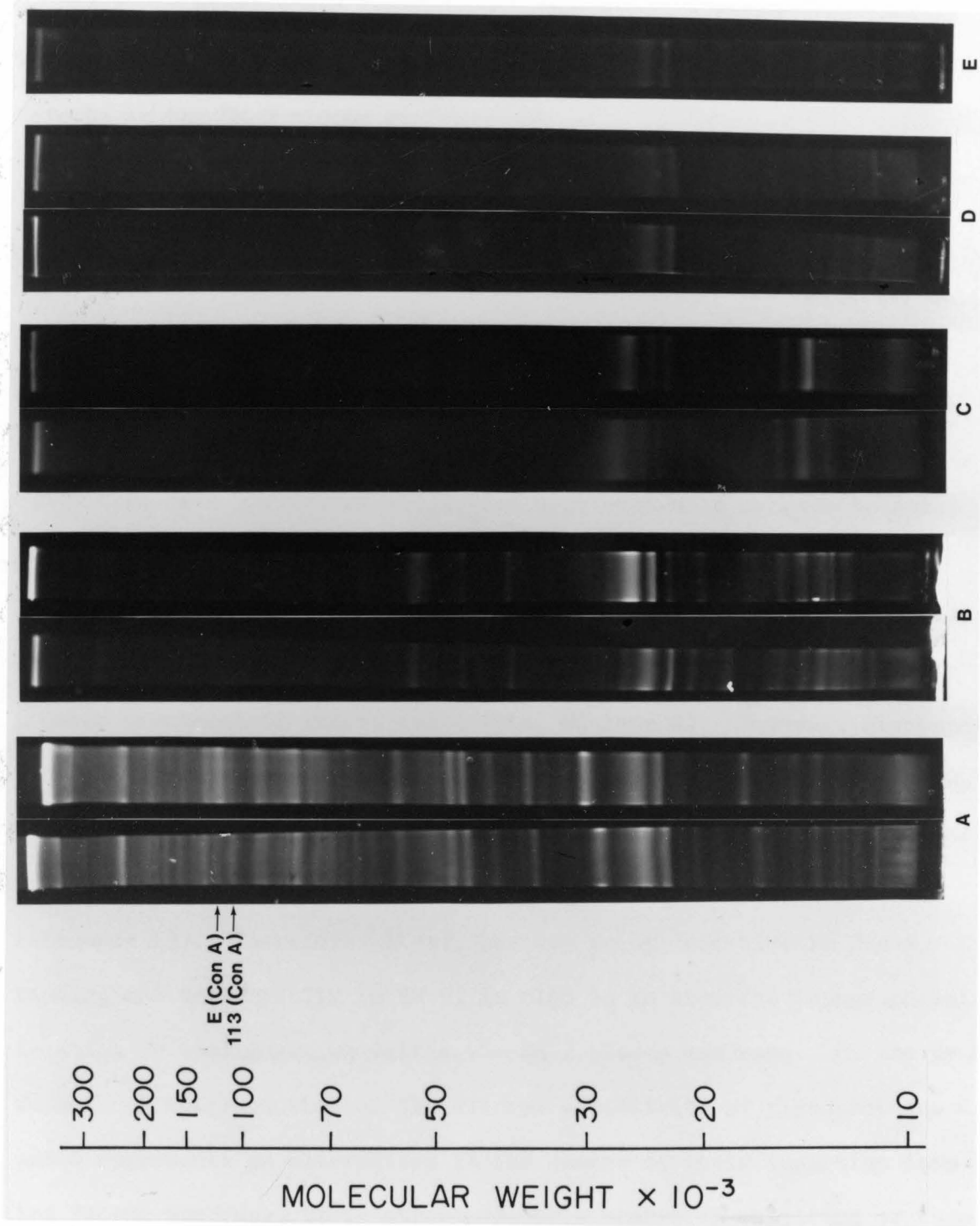
Carbohydrates	A3	HM 2
Fucose	0.039 ± 0.012	0.076 ± 0.027
Mannose	0.036 ± 0.001	0.039 ± 0.010
Glucose	0.027 ± 0.001	0.030 ± 0.005
Glucosamine	0.122 ± 0.013	0.124 ± 0.005
Galactose	<0.005	<0.005
Galactosamine	<0.001	<0.001
Sialic Acids	<0.001	<0.001

Values given are mean ± standard deviation.

studied with the following FITC-lectins: Con A, WGA, FBP, and RCA 60 in order to determine if differences in the carbohydrate moieties of glycoproteins could be detected. The monosaccharide inhibitors for these lectins are respectively: D-mannose or D-glucose, N-acetyl-D-glucosamine, L-fucose, and D-galactose or N-acetyl-D-galactosamine. This method can detect about 10^4 receptors per cell (16) and is therefore more sensitive than the analysis of sugars by gas-liquid chromatography. With the exception of FBP, the lectin binding patterns shown here are inhibited by the appropriate monosaccharide haptens (16). There are two differences in lectin receptors between A3 and HM 2 detectible with Con A (Fig. 7A). Receptor E(Con A) binds more lectin in HM 2, while receptor 113(Con A) binds more Con A in A3. The difference in lectin binding to E(Con A) was apparent over a wide range of concentrations of Con A. In other experiments with a concentration of Con A 5 times the standard amount, the difference in E(Con A) was accentuated and the difference was still obvious at 1/5 the routine concentration of Con A (data not shown). Since antigenic and Con A binding differences between A3 and HM 2 cells both comigrated with the major glycoprotein of vegetative plasma membranes E(gp), we purified this molecule and found that, indeed, these differences are associated with the same molecule.[6]

No difference between A3 and HM 2 plasma membranes were detected with the other lectin used (Fig. 7B-E). This indicates that extensive modification of glycoprotein side chains has not occurred in HM 2.

Fig. 7. Identification of lectin receptors in vegetative A3 and HM 2 plasma membranes. A3 (left lane of each pair) and HM 2 (right lane of each pair) plasma membrane glycoproteins were resolved by SDS gel electrophoresis, fixed in the gels and reacted with fluorescent lectin as described in the Methods. The lectins used were A) Con A; B) FBP; C) WGA; D) RCA-60; and E) no lectin (autofluorescence control). The major differences in Con A receptors are indicated as E (Con A) because this receptor comigrates with E(gp) (13), and 113 (Con A) because the molecular weight of this receptor is 113,000.


E
D
C
B
A
E (Con A)
113 (Con A)
300
200
150
100
70
50
30
20
10
MOLECULAR WEIGHT × 10⁻³

Plasma membrane glycoprotein composition and topography

HM 2 plasma membranes contain defective glycoproteins as detected by Con A binding and immunology. Therefore, a third, independent technique for identifying glycoproteins was used to search for additional defects in the HM 2 plasma membrane.

PAS staining of plasma membranes indicated 2 additional differences in HM 2 (Fig. 8 and 9). J(gp) and K(gp) are present in lesser amounts in the HM 2 plasma membrane. Although E(gp) was defective antigenically and in Con A binding in HM 2, it was not altered in PAS staining. Therefore, the defect in E(gp) in HM 2 does not significantly affect the number of periodate oxidizable carbohydrate residues.

We have previously[2] (13) used treatment with pronase to assay the topography of plasma membrane molecules during development. Topographical differences between A3 and HM 2 PAS positive glycoproteins are also detectable. As in A3 (13) no glycoproteins are sensitive to pronase treatment of intact cells (Fig. 8, lane 2). However, glycoproteins C, E, and G are sensitive to pronase treatment of isolated HM 2 plasma membranes (Fig. 8, lane 3), although they are relatively insensitive to similar treatment of A3 plasma membranes (Fig. 4, lane 8, reference 13). Therefore, E(gp), besides being defective in Con A binding and antigenicity in HM 2, is also in an aberrant topographical location or configuration within the HM 2 plasma membrane. At the present we do not know whether the altered sensitivity of glycoproteins C and G represents an alternation in the nature of their insertion into the plasma membrane, their structure, or a change in shielding of these components by an altered molecule such as E(gp).

Fig. 8. SDS gel electrophoresis of plasma membrane glycoproteins from vegetative wild-type and mutant cells identified with the PAS method.

Lanes from left to right are:

1) Vegetative HM 2 plasma membranes;

2) Plasma membranes from pronase treated vegetative HM 2 cells;

3) Pronase treated vegetative HM 2 plasma membranes;

4) As in 1;

5) Vegetative A3 plasma membranes.

White letters indicate glycoproteins deficient in HM 2. Black letters indicate glycoproteins sensitive to pronase treatment of isolated HM 2 plasma membranes that were insensitive to similar treatment in A3 (13). The lettering system is the same as used in reference 13.

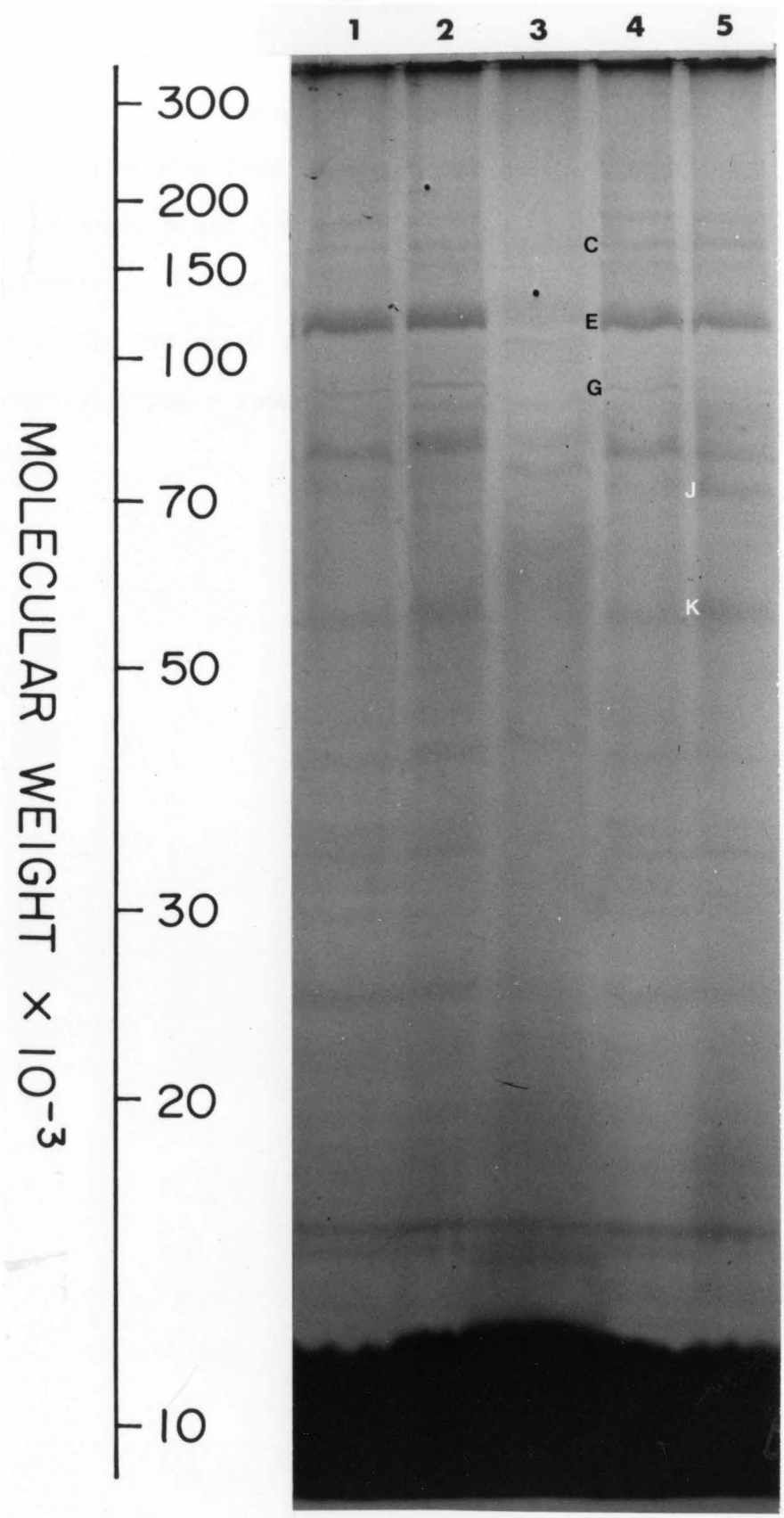
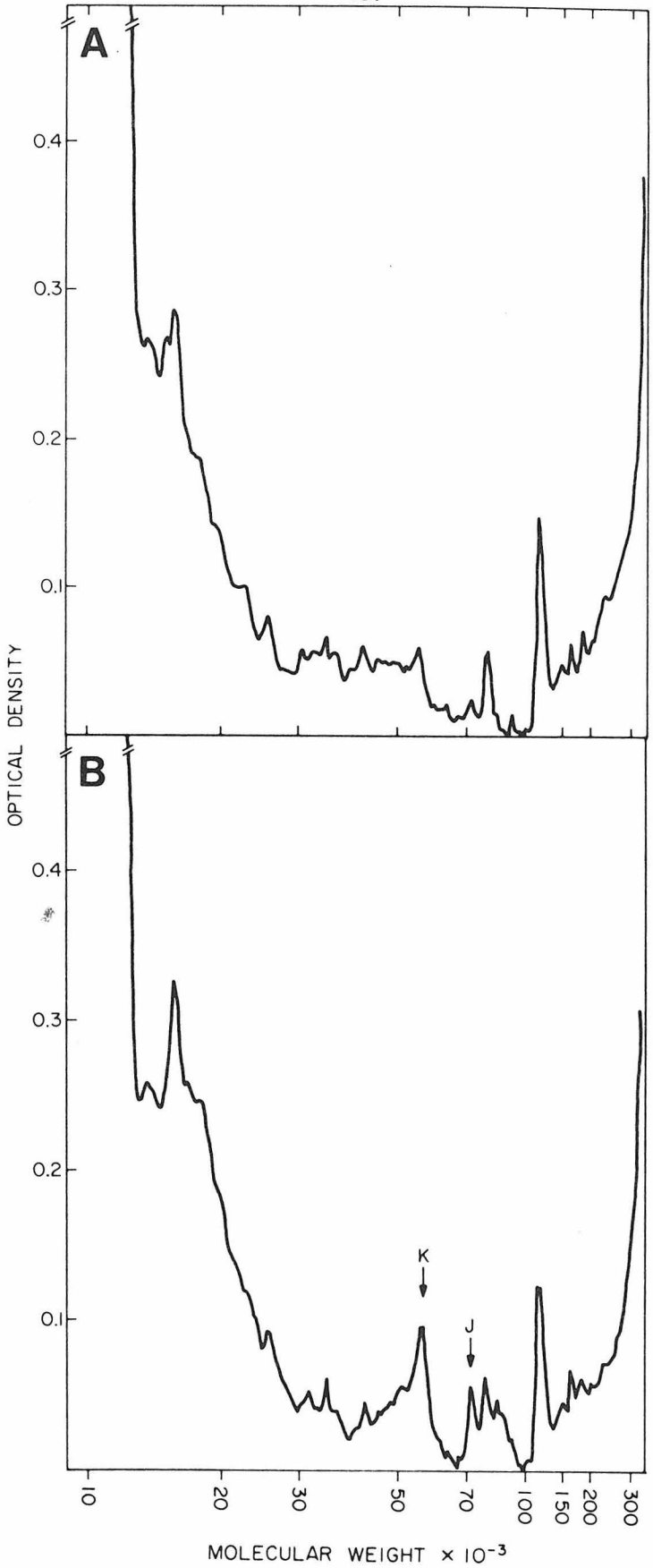

1
2
3
4
5
300
200
150
100
70
50
30
20
10
C
E
G
J
K
MOLECULAR WEIGHT × 10⁻³

Fig. 9. Densitometric scans of PAS staining intensity of plasma membrane glycoproteins from wild-type and mutant cells. Photographic negatives of lanes 4 and 5 from Fig. 8 were scanned on a Syntex AD-1 Autodensitometer. A) lane 4 (HM 2 plasma membranes); B) lane 5 (A3 plasma membranes). The two major differences, J(gp) and K(gp) are indicated. The apparent difference about molecular weight 85,000 daltons was not reproducible.


A
B
0.4
0.3
0.2
0.1
OPTICAL DENSITY
K
J
10
20
30
50
70
100
150
200
300
MOLECULAR WEIGHT × 10⁻³

Plasma membrane polypeptide composition and topography

We were concerned with the possibility that the defects observed above in the HM 2 plasma membrane were secondary effects of a general disruption in plasma membrane composition or organization. Therefore, plasma membrane composition and topography were examined.

No significant differences were found between A3 (Fig. 10, lane 5) and HM 2 vegetative plasma membranes (Fig. 10, lane 4) in polypeptide composition or cell surface exposure of polypeptides as determined by pronase treatment of intact cells (compare Fig. 10, lane 2 with Fig. 3, lane 2, reference 13). As previously observed in HM 2 cells incubated for development,[2] the number of polypeptides resistant to pronase treatment of purified HM 2 plasma membranes (Fig. 10, lane 3) is less than in similarly treated A3 cells (Fig. 3, lane 3, reference 13).

On 2-dimensional gels (isoelectric focusing followed by SDS gel electrophoresis) loaded with 9 times as much plasma membrane protein (allowing the resolution of 95 polypeptides versus the 55 seen after one-dimensional SDS gel electrophoresis), no major repeatable differences in polypeptide composition between A3 (Fig. 11A) and HM 2 plasma membranes polypeptides (Fig. 11B) could be observed.

Plasma membrane and whole cell lipid composition

Since defects in several glycoproteins occur in HM 2, we thought it possible that glycolipids might also be defective. Therefore, we examined plasma membrane lipid composition. HM 2 plasma membranes contain a glycolipid not found in A3 plasma membranes (Fig. 12, lane 6). Since it has a similar R_f to cerebroside (data not shown) and since it partitions into the lower phase (20) it may be cerebroside but it has not

Fig. 10. SDS gel electrophoresis of plasma membrane polypeptides from vegetative wild-type and mutant cells.

Lanes from left to right are:

1) Vegetative HM 2 plasma membranes;

2) Plasma membranes from pronase treated vegetative HM 2 cells;

3) Pronase treated plasma membranes from vegetative HM 2 cells;

4) As in 1.

5) Vegetative A3 plasma membranes.

Letters indicate polypeptides sensitive to pronase treatment of intact HM 2 cells. The same lettering system is used as in reference 13.

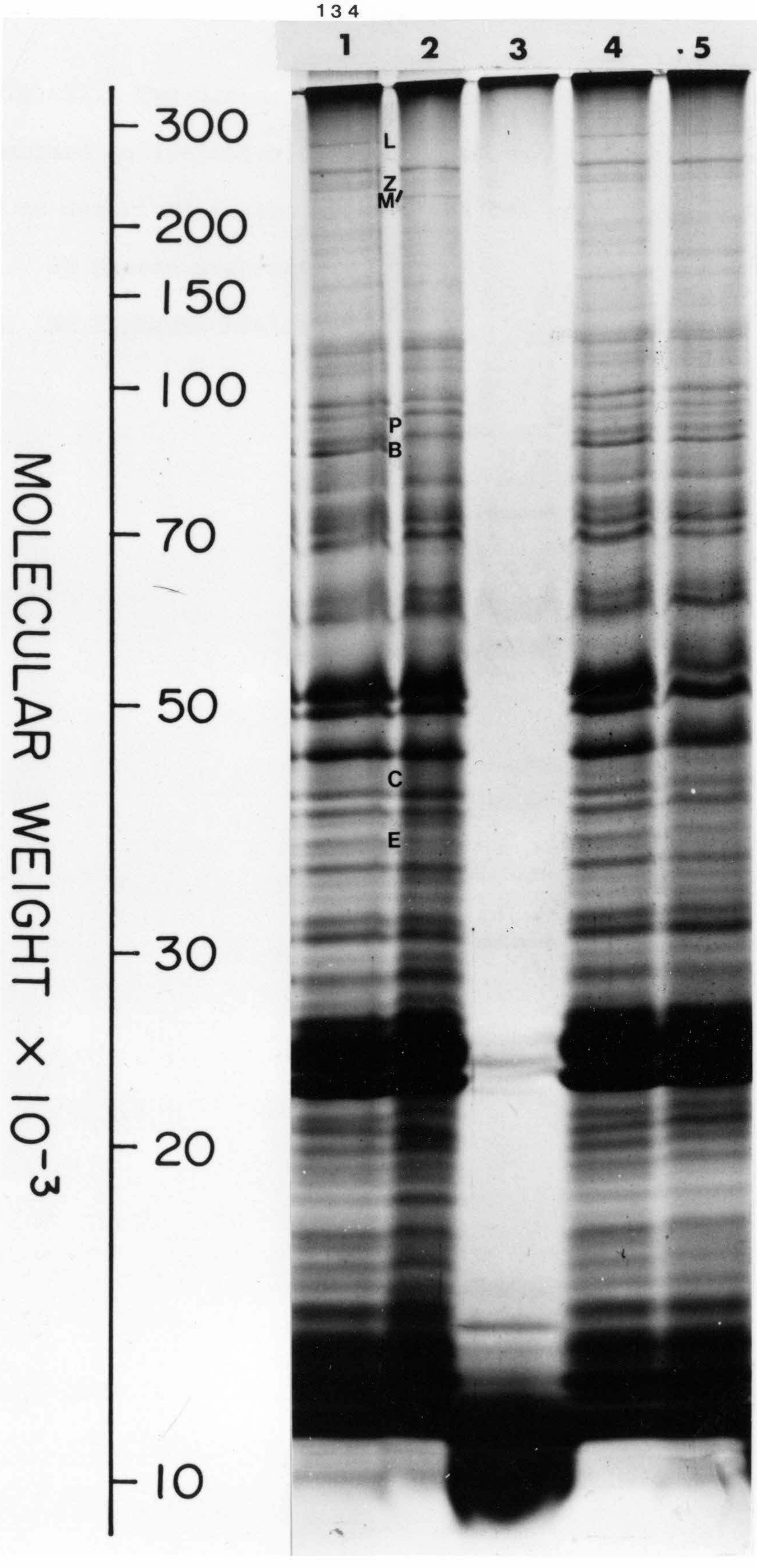

1
2
3
4
5
300
200
150
100
70
50
30
20
10
MOLECULAR WEIGHT × 10⁻³
L
Z
M
P
B
C
E

Fig. 11. Two-dimensional gel electrophoresis of A3 and HM 2 plasma membrane polypeptides. Two-dimensional gel electrophoresis was performed as described in the Methods and the gels stained for protein.

A. A3 plasma membranes.

B. HM 2 plasma membranes.

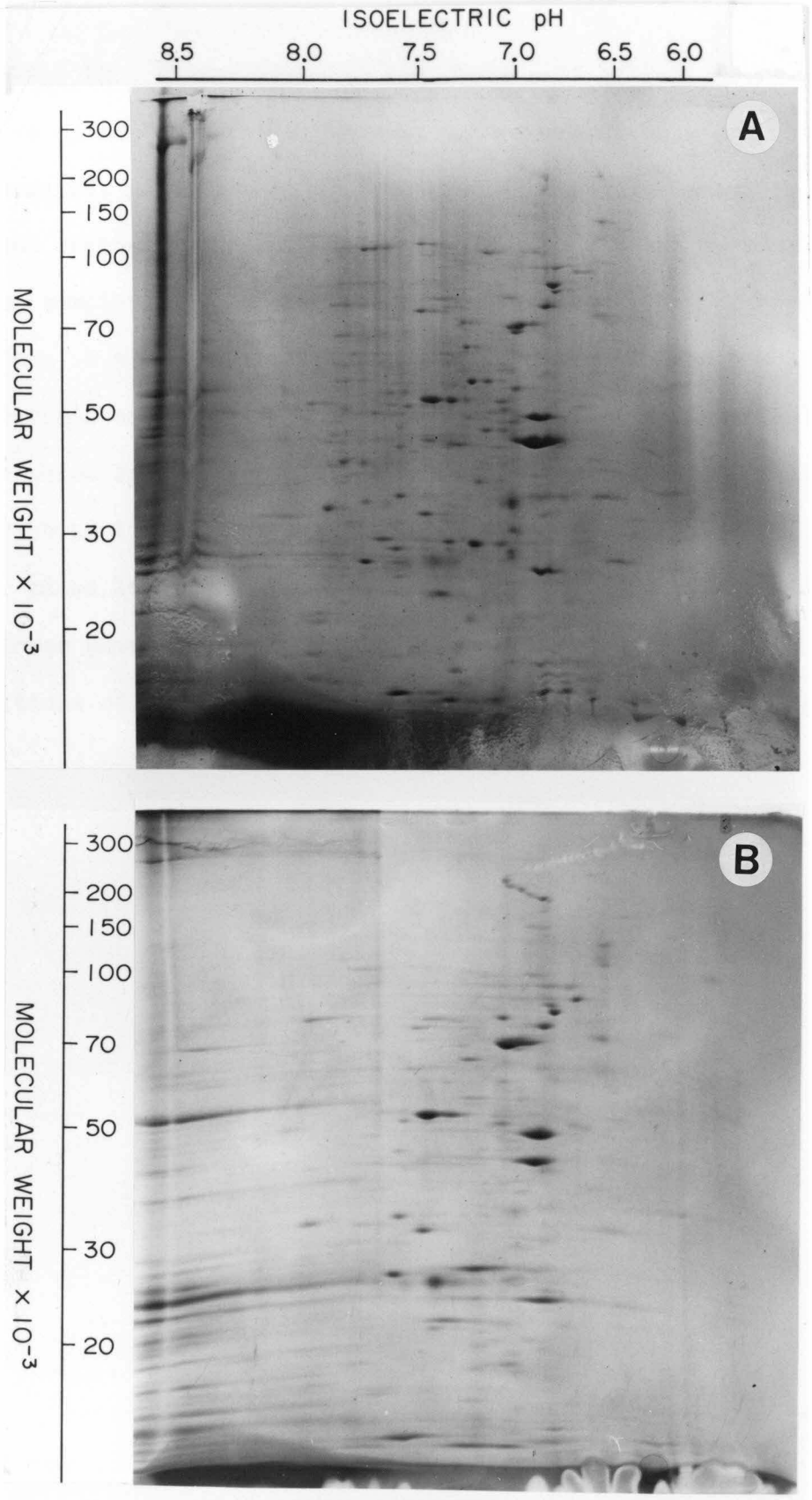
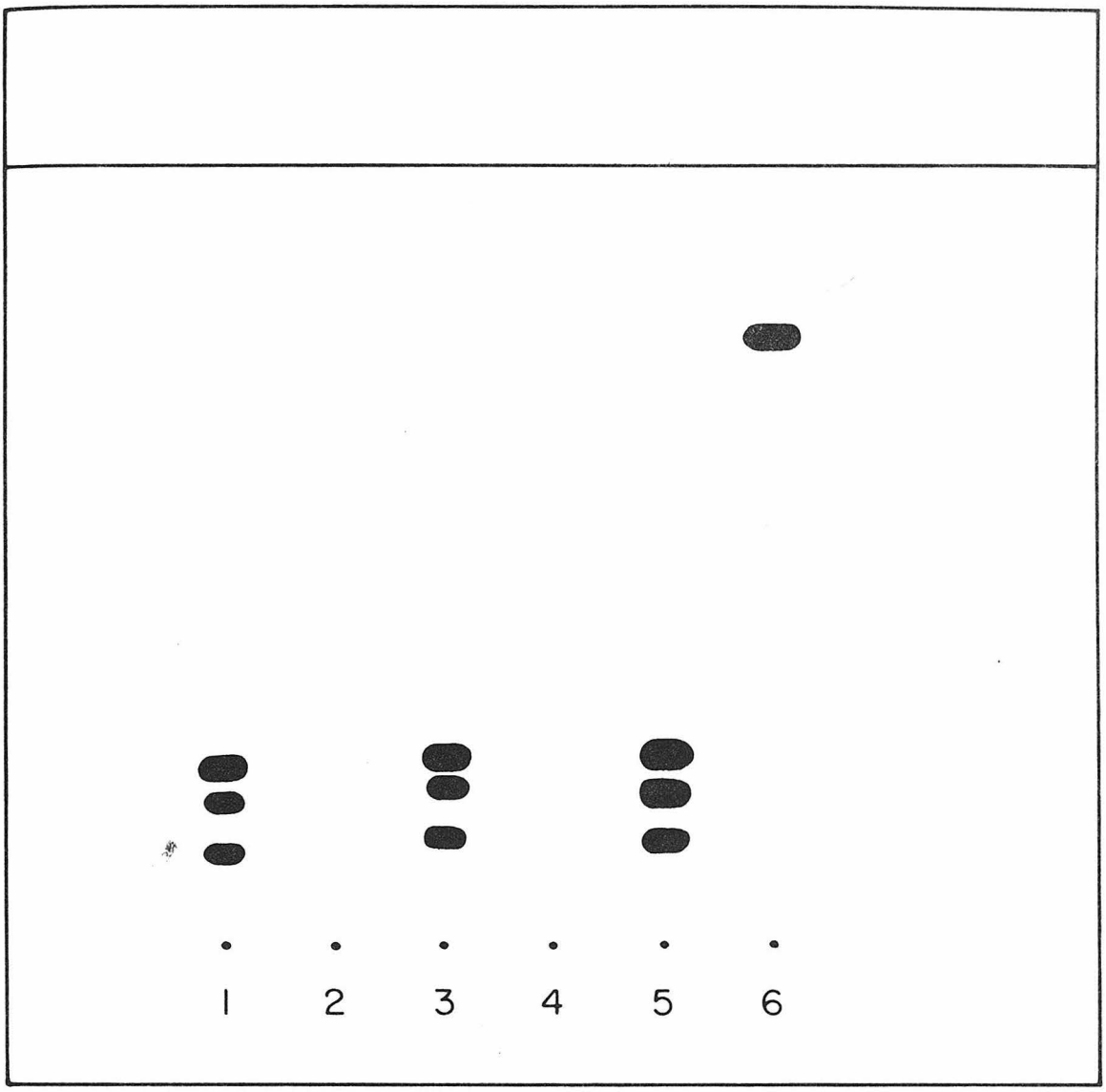

ISOELECTRIC pH
8.5
8.0
7.5
7.0
6.5
6.0
A
300
200
150
100
70
50
30
20
MOLECULAR WEIGHT × 10⁻³
B
300
200
150
100
70
50
30
20
MOLECULAR WEIGHT × 10⁻³

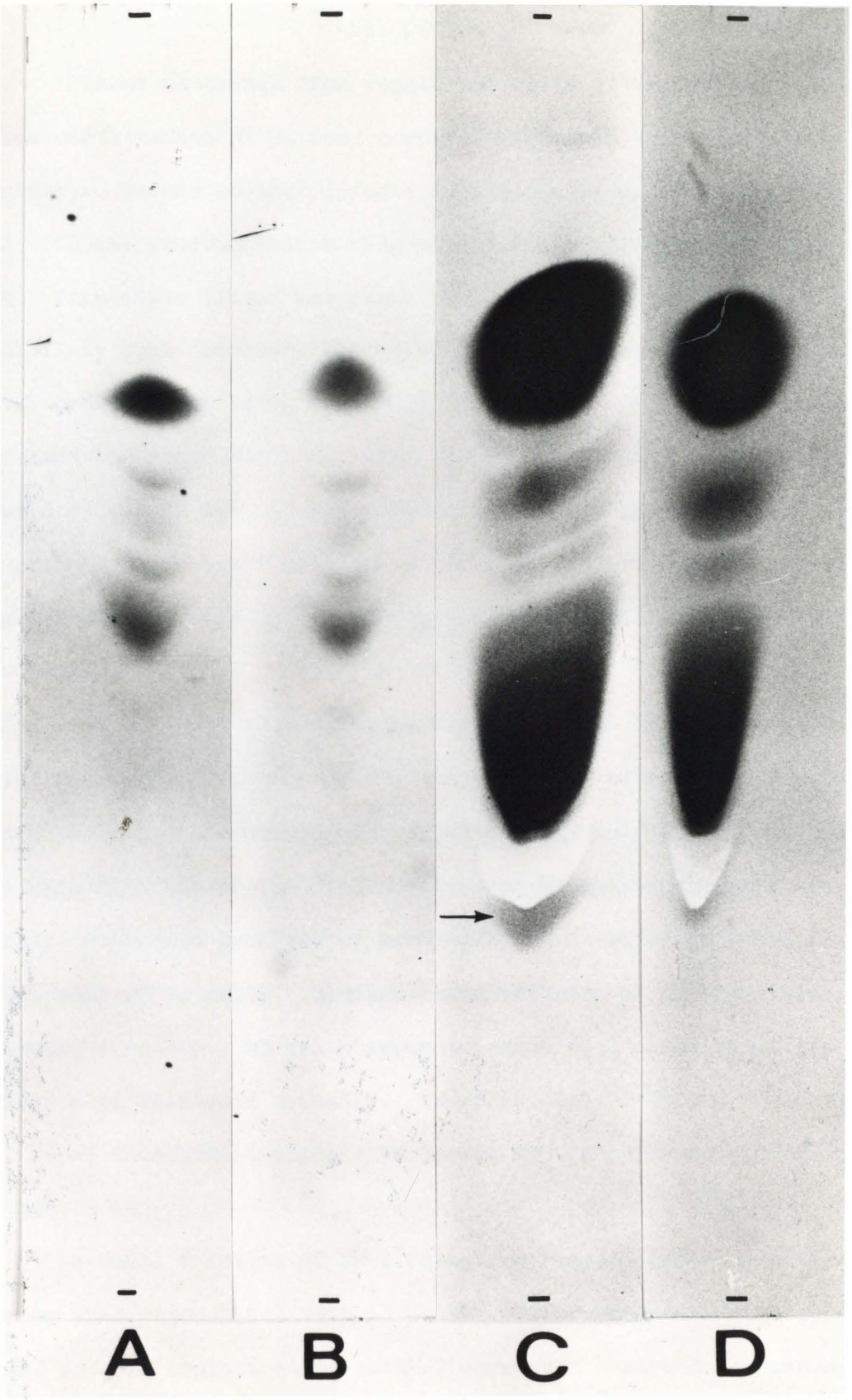
Fig. 12. Plasma membrane and whole cell glycolipids from vegetative A3 and HM 2 cells. Plasma membrane and whole cell lipids were extracted, partitioned, chromatographed on silica gel thin layer plates and stained for carbohydrate as described in the Methods. The following samples were run and the results traced: 1) Upper phase lipids from 2×10^8 A3 cells; 2) upper phase lipids from 4×10^6 A3 cells (protein equivalent of plasma membrane from 2×10^8 A3 cells); 3) upper phase lipids from plasma membranes from 2×10^8 A3 cells; 4) Lower phase lipids from plasma membranes from 2×10^8 A3 cells; 5) upper phase lipids from plasma membrane from 2×10^8 HM 2 cells; and 6) lower phase lipids from plasma membranes from 2×10^8 HM 2 cells. The positions of the sample origins and solvent front are indicated.


1
2
3
4
5
6

been characterized. The remaining glycolipids found in both A3 (Fig. 12, lane 3) and HM 2 (Fig. 12, lane 5) probably have more extensive carbohydrate domains since they partition primarily into the upper phase (30) (in some preparations a minor portion partitioned into the lower phase) and have smaller R_f's indicating they are more polar. Therefore it is possible that the HM 2 specific glycolipid is an incomplete form of the glycolipids common to both A3 and HM 2. These low R_f glycolipids are found only in the plasma membrane as would be expected if the lipids bear complex oligosaccharide chains (30). Whole cells and plasma membranes from a similar number of cells contain a similar amount of glycolipids (Fig. 12, lanes 1 and 3). Glycolipids were below detection (Fig. 12, lane 2) in the whole cell protein equivalent to the protein content of the plasma membranes shown in Fig. 12, lane 3. Although the low R_f glycolipids are identical in lipid preparations prepared in parallel from A3 and HM 2 plasma membranes, they are extremely sensitive to slight differences between preparations. For example, heating samples to 50° during rotary evaporation of the upper phase destroys these glycolipids.

No differences in phospholipid composition are apparent between A3 (Fig. 13A) and HM 2 (Fig. 13B) plasma membranes. In contrast to the glycolipids, most of the cell's mass of phospholipids is not in the plasma membrane. The whole cell phospholipid content of A3 (Fig. 13C) or HM 2 (Fig. 13D) cells is much greater than the phospholipid content of plasma membranes from an equivalent number of A3 (Fig. 13A) or HM 2 (Fig. 13B) cells. There appears to be a slight quantitative difference in one phospholipid between A3 and HM 2 whole cells.

Fig. 13. Plasma membrane and whole cell phospholipids from vegetative A3 and HM 2 cells. Plasma membrane and whole cell lipids were extracted, chromatographed on silica gel thin layer plates, and stained for phospholipids as described in the Methods. The following samples were run: A) Total lipids from plasma membranes from 1 x 10^8 A3 cells; B) total lipids from plasma membranes from 1 x 10^8 HM 2 cells; C) total lipids from 1 x 10^8 A3 cells; and D) total lipids from 1 x 10^8 HM 2 cells. The arrow indicates a quantitative difference in whole cell phospholipid composition between A3 and HM 2. The position of the sample origins and solvent front are indicated.


A
B
C
D

DISCUSSION

Plasma membranes from vegetative cells of the spontaneous aggregation minus mutant HM 2, were compared with wild type A3 plasma membranes in order to deduce whether defects in the plasma membrane might be associated with the genetic lesion responsible for the inhibition of development. Vegetative plasma membranes were examined to reduce the possibility that secondary differences due to inhibition of the developmental program were being observed. The use of a spontaneous mutant decreases the probability of multiple mutations complicating the results. Therefore, one or more of the defects detectible in HM 2 plasma membranes are probably associated with the inhibition of development. However, only genetic analysis of the mutant can unequivocally resolve this question.

Two biological experiments suggested that HM 2 has a defective cell surface. Coincubation of HM 2 and A3 cells at a ratio of 3:1 or higher inhibited the development of A3 cells.. This effect was not due to simple dilution of the concentration of A3 cells present or to diffusible molecules produced or removed by HM 2 cells which inhibited development of A3 cells. Similar concentrations of A3 cells plated alone developed normally. A3 cells separated from HM 2 cells by millipore filters also developed normally. Therefore, the inhibitory effect of HM 2 on A3 development appears to depend on the opportunity for cell contact.

A small fraction of HM 2 cells are rescued in the ability to develop when coincubated with A3 cells. This phenomenon also appears to depend on cell contact since no HM 2 were even observed to develop

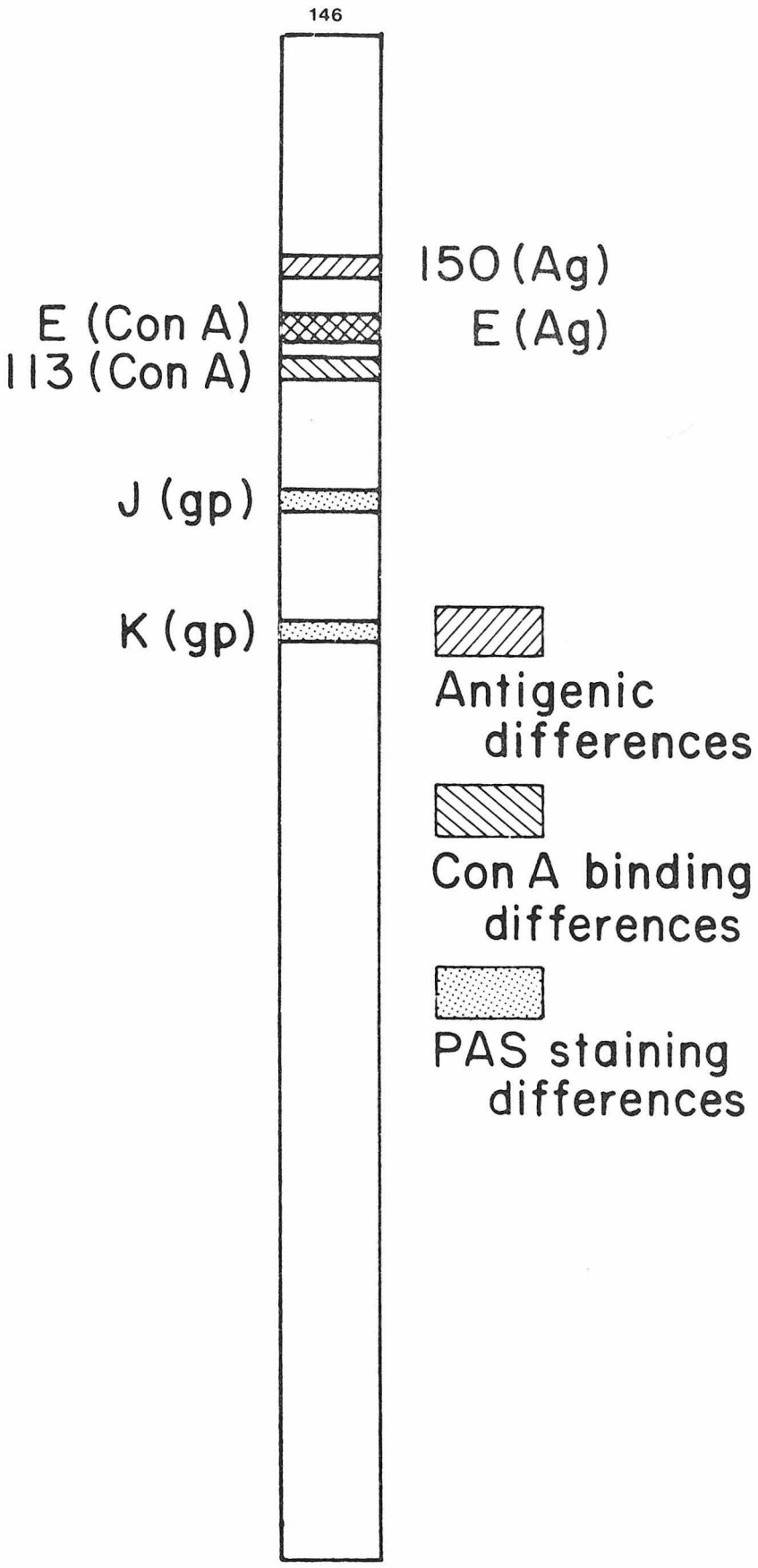
normally when incubated alone or in a transfilter orientation from A3 cells. Similar phenomena have previously been observed with other mutants. Ennis and Sussman have found two mutants that inhibited the development of wild type cells (5) when coincubated together, but not when incubated on opposite sides of a 30 μ thick agar membrane. Earlier, Sussman had found that aggregation minus mutants could participate in development even to the point of becoming spores if paired in the proper synergistic relationship (6). Unfortunately, the mutants used in the latter study were not used in this prior study so we do not know if these two phenomena are always coupled or if HM 2 is unique in this regard.

The cell surface of HM 2 is not only defective in the amoebae but is clearly defective during development. When incubated alone, HM 2 cells do not collect into aggregates or form the cohesion-mediating contact sites A. Concurrently, we have detected the occurence of several defects in plasma membrane composition and topography. Several normal development changes in plasma membrane composition and topography do not occur in HM 2.[2] These aberrations include the absence of changes in the amount of some of the macromolecules identified as defective in vegetative HM 2 plasma membranes in this study which normally occur during slime mold development. For instance, E(gp) is not detectible in preculmination A3 plasma membranes, however, it is still present in similarly incubated HM 2 cells. Therefore, defects are constantly present in the HM 2 cell surface that may continue to abnormally participate in the interaction between A3 and HM 2 cells inhibiting the development of A3 cells.

As suggested by the biological experiments, the cell surface of vegetative HM 2 cells is defective in several molecules. The macromolecular defects we have detected in the HM 2 plasma membrane are summarized in Fig. 14. Two antigens, present on the A3 cell surface, as demonstrated by the ability of intact A3 cells to adsorb antibodies with these specificities, are defective in structure or expression on HM 2 cells. One of these, 150(Ag), may only be present in decreased amount or cryptic in HM 2. The other, E(Ag), although present at the cell surface, must be altered in structure in HM 2 since HM 2 adsorbed anti-A3 plasma membrane antiserum binds to E(Ag) in A3 but not in HM 2. HM 2 E(Ag) does bind unadsorbed antiserum, in fact, it binds more antibody from unadsorbed anti-A3 plasma membrane antiserum than does A3 E(Ag).

Two independent models have been constructed for the general nature of the antigenic relationships between E(Ag) and 150(Ag) from A3 and HM 2 cells. Other, more complicated, but supportable models are possible. In the first model, glycoprotein E from A3 cells is suggested to bear a unique antigen not found elsewhere on A3 cells or at all on HM 2 cells. Glycoprotein E in HM 2 cells bears an antigen that is also found on another molecule (X) in HM 2 cells and on X in A3 cells. The 150(Ag) is structurally similar in A3 and HM 2 cells but a greater amount of the antigen is exposed on A3 cells. Therefore, A3 adsorption removes antibody against the A3 E(Ag), the X antigen, and the 150(Ag). HM 2 adsorption removes antibody against the X antigen (or HM 2 E(Ag)) and a small part of the antibody against the 150(Ag). The model thus predicts that A3 adsorption will remove all antibodies against glycoprotein E and the 150,000 dalton macromolecule, while HM 2 adsorption will

Fig. 14. Macromolecular differences in A3 and HM 2 plasma membrane composition. The differences found in this paper between A3 and HM 2 plasma membrane macromolecules are summarized schematically in a mock gel lane.


150 (Ag)
E (Con A)
E (Ag)
113 (Con A)
J (gp)
K (gp)
Antigenic differences
Con A binding differences
PAS staining differences

leave antibodies against the glycoprotein E in A3 cells and the 150,000 dalton macromolecule in A3 and HM 2 cells.

The second model suggests that both macromolecules detectable on A3 plasma membranes by HM 2 adsorbed serum (glycoprotein E and the 150,000 dalton macromolecule) bear a common antigen (the 150-E antigen) and that glycoprotein E from A3 cells has an additional unique antigenic site (the E antigen) in close proximity to the location of the 150-E site on E(gp). In this model, glycoprotein E from A3 and HM 2 cells bear the E antigen in common. In addition, glycoprotein E from A3 cells has an antigen which is found only on the 150,000 dalton macromolecule of HM 2 cells (the 150-E antigen). Since there appears to be less 150,000 dalton macromolecule exposed in HM 2 than in A3 cells, A3 adsorption is assumed to remove antibody against both of these antigens, while HM 2 adsorption leaves most of the antibody against the 150-E antigen.

We also detected several defects in HM 2 using techniques sensitive only to carbohydrate structure. Two Con A receptors were different in A3 and HM 2 plasma membrane. E(Con A), although detectable in A3, bound more lectin in HM 2. 113(Con A) was detectable only in A3. No differences were detected between A3 and HM 2 plasma membrane in receptors for the lectin WGA RCA 60, or FBP. Two PAS positive glycoproteins were diminished in amount in HM 2. Three other PAS positive glycoproteins, including glycoprotein E, which was also detected as an antigenic and Con A binding defect, were more sensitive to pronase treatment of isolated plasma membrane in HM 2 than in A3. A glycolipid was present in HM 2 that was not detectable in A3. This glycolipid may be an incomplete form of more complex glycolipids common to both A3 and HM 2 but must be examined in more detail.

On the other hand, the polypeptide and phospholipid compositions of A3 and HM 2 plasma membranes were identical. Therefore, the differences we have observed between A3 and HM 2 plasma membrane proteins in antigenicity, Con A binding, and PAS staining must be due either to modifications that do not effect mobility on SDS gels or to minor plasma membrane components. These studies also indicate that an examination of plasma membrane composition only by one or two methods may mask many important independent structural defects. To survey fully plasma membrane composition, a variety of independent analytical techniques may generally be necessary.

With one exception, the defects found in the HM 2 plasma membrane were detectable only with one of the techniques used. This may be due to different structural requirements for antigenicity, lectin binding, and the PAS reaction. Therefore, 113(Con A) J(gp), and K(gp) may not be antigenic in rabbits or may share their antigenic sites with other membrane molecules and so would not be observed with adsorbed sera. Antibody binding to J(gp) and K(gp) by unadsorbed serum also might have been obscured by antibody binding to the broad, continuous band of antigen observed. The defects in 150(Ag), E(Con A), 113(Con A) need not involve changing the number of PAS positive carbohydrate residues on a glycoprotein. The fact that no differences in lectin binding were seen at the position of J(gp) and K(gp) suggests that although fewer PAS positive carbohydrate residues are present in HM 2 than in A3, an equal number of lectin binding sites with equal affinities are available. Alternatively, they may only be comigrating with other lectin receptors.

E(gp) may bear an oligosaccharide chain(s) terminating in α-D-mannose or α-D-glucose, while A3 E(gp) may have one additional carbohydrate molecule not recognized by Con A at the end of an otherwise identical carbohydrate chain. This configuration could cause A3 and HM 2 E(gp) to be antigenically distinct and cause E(gp) from HM 2 cells to bind more Con A than E(gp) from A3 cells.

Almost all the defects apparent in the HM 2 vegetative plasma membrane appear to be in carbohydrate-containing molecules. Analogous defects are frequently associated with the transformed state of cells (38). Recently, defective glycosylation of cell surface glycoproteins was directly related to defects in adhesion to substratum, cell shape, number of microvilli, Con A agglutinability, and mobility in 3T3 cells (39) by Pouysségur and Pastan. In that study, defective glycosylation was related to a defect in glucosamine metabolism. Similar structural defects in HM 2 glycoproteins may be responsible for its inability to develop. The primary defect in HM 2 may be in the activity or substrate specificity of a glycosyl transferase (a defect frequently found in transformed cells (38)), a glycosidase or in an alteration in the cellular concentration of a precursor. Such a defect could alter the structure of oligosaccharide containing molecules in HM 2.

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FOOTNOTES

[1] This paper is number six in a series entitled "The Role of the Plasma Membrane in the Development of Dictyostelium discoideum." Paper number 5 is footnote 2.

[2] Hoffman, S., and McMahon, D., submitted for publication.

[3] Abbreviations are as follows: Con A, concanavalin A; WGA, wheat germ agglutinin; FBP, L-fucose binding protein (Ulex europeus agglutinin I); RCA-60, Ricinus communius agglutinin 60; SDS, sodium dodecyl sulfate; PAS, periodic-acid Schiff; FITC, fluorescein isothiocyanate conjugated. E(Ag) is read antigen E. E(Con A) is read Con A receptor E. E(gp) is read glycoprotein E.

[4] McMahon, D., unpublished results.

[5] Wrann, M., and Todd, C. W., submitted for publication.

[6] Hoffman, S., and McMahon, D., submitted for publication.

[7] Hoffman, S., and McMahon, D., unpublished results.

CHAPTER V

THE MAJOR GLYCOPROTEIN OF THE PLASMA MEMBRANE OF AMOEBAE OF *DICTYOSTELIUM DISCOIDEUM*: CHARACTERIZATION AND READDITION TO THE PLASMA MEMBRANE USING LIPID VESICLES

THE MAJOR GLYCOPROTEIN OF THE PLASMA MEMBRANE OF AMOEBAE OF *DICTYOSTELIUM DISCOIDEUM*: CHARACTERIZATION AND READDITION TO THE PLASMA MEMBRANE USING LIPID VESICLES.

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Running title: *D. discoideum* plasma membrane glycoprotein

ABSTRACT

The major glycoprotein, E(gp), of vegetative D. discoideum plasma membranes was purified from wild type (A3) cells and cells of an aggregation minus mutant (HM 2) by preparative SDS gel electrophoresis. E(gp) is homogeneous on SDS gels made with an exponential gradient of polyacrylamide and forms a broad band, all of which binds concanavalin A upon isoelectric focusing. E(gp) from HM 2 cells binds more concanavalin A than E(gp) from A3 cells. The amino acid composition of E(gp) is noteworthy for the high content of polar residues present although the molecule is an intrinsic membrane glycoprotein and is insoluble in water. E(gp) contains glucosamine and mannose and may contain glucose but a variety of other sugars were not detected. E(gp) is expressed at the cell surface of about two-thirds of a population of vegetative A3 cells as determined by indirect immunofluorescence.

E(gp) in lipid vesicles is incorporated by cells. A large portion of previously incorporated exogenous E(gp) is lost by A3 or HM 2 cells incubated for development. This loss occurs later in A3 cells than in HM 2 cells. In contrast to these results, growing vegetative cells do not lose added exogenous E(gp). Cycloheximide treated cells initially incorporate E(gp), but then lose all of the exogenous glycoprotein. E(gp) in lipid vesicles apparently becomes associated with the plasma membrane of target cells by a mechanism which involves either fusion of the vesicles with the plasma membrane or direct transfer of E(gp) from vesicle to cell.

The association of E(gp) with the cell surface has been confirmed by immunofluorescence.

INTRODUCTION

The molecular composition of the D. discoideum plasma membrane changes during development concurrently with changes in plasma membrane function (30). Although about 40% of the polypeptide species change in amount present (17), changes in glycoprotein composition are more extensive. Twenty-four of 25 periodic acid-Schiff (PAS) positive glycoproteins change in amount present during development (17). West and McMahon showed that at least 80% of concanavalin A (Con A) receptors and all wheat germ agglutinin (WGA) receptors also change in amount present during development (46,47). Geltosky et al. (9), showed a lesser change in the spectrum of Con A receptors using a different method for identifying receptors. Defects in glycoproteins may be correlated with the inability of a mutant to participate in normal developmental interactions[1].

One developmentally controlled glycoprotein is E(gp), the major PAS positive glycoprotein of vegetative D. discoideum plasma membranes (17). E(gp) has been identified as a Con A receptor[1] (46). E(gp) is mobile in the plane of the membrane since all Con A receptors have been shown to be mobile in the D. discoideum plasma membrane (33). Although located at the cell surface of A3 cells as evidenced by the ability of intact cells to adsorb out all antibody against this molecule[1], E(gp) is insensitive to pronase treatment of intact cells (17). Furthermore, E(gp) is partially resistant to pronase treatment of isolated A3 plasma membranes. This suggests that E(gp) is buried in the membrane

and that exposed parts of the molecule, if any, are shielded from pronase, perhaps by carbohydrates. The amount of E(gp) decreases during development from its highest level in vegetative A3 plasma membranes to an intermediate level in early aggregation[2] and late aggregation (16) plasma membranes declining to an undetectable level in preculmination plasma membranes (17). In the HM 2 mutant, E(gp) decreases during incubation to the intermediate level but no further[2] after incubation in conditions which lead to normal development of A3 cells. If development is blocked by cycloheximide treatment of A3 cells, E(gp) decreases little from the vegetative level even after 18 h incubation[2].

E(gp) has been shown to be structurally aberrant in plasma membranes of an aggregation defective mutant of D. discoideum, HM 2. Although E(gp) is similar in A3 and HM 2 vegetative plasma membranes in PAS staining intensity, it binds more Con A and is antigenically different on glutaraldehyde-fixed SDS gels of HM 2 plasma membranes[1]. As in A3 cells, E(gp) is resistant to pronase treatment of intact HM 2 cells. However, unlike in A3, E(gp) is very sensitive to pronase treatment of isolated HM 2 plasma membranes[1]. The altered properties of the plasma membrane of HM 2 have allowed preparation of an antiserum directed against E(gp) and another molecule with an apparent molecular weight of 150,000 daltons.

The major cell surface glycoprotein (CSP) of chick embryo fibroblasts has been directly added to transformed cells lacking this molecule and has been found to partially restore normal

morphology, adhesiveness, and contact inhibition of movement (49). Added CSP can be recovered from treated cells indicating that this molecule becomes associated with target cells. Since most plasma membrane macromolecules are hydrophobic (6), fusion of lipid vesicles (containing the macromolecule in question embedded in their lipid bilayer) with intact cells presents another method that may be more generally applicable for adding plasma membrane macromolecules to cells. Several plasma membrane macromolecules have been incorporated into lipid vesicles (12,14,15,18,31,32,38). However, although several workers have incorporated phospholipid vesicles into the plasma membrane of target cells (3,28,34,35,37), no previous report has demonstrated the transfer of plasma membrane macromolecules from one cell line to another via lipid vesicles. Soluble immunoglobulin (27) and horseradish peroxidase (26) were apparently incorporated into the plasma membrane of target cells in an active form indicating the feasibility of this approach for incorporation of proteins into the plasma membrane.

In this paper, we report the purification and partial characterization of E(gp). This cell surface glycoprotein, purified from one strain of D. discoideum, can be transferred to the plasma membrane of another strain via lipid vesicles. Transfer of plasma membrane macromolecules via lipid vesicles has no apparent deleterious effects on the target cells. The characteristics of the transfer process have been investigated since this method may prove useful for the analysis of the function of the plasma membrane in the

development of D. discoideum.

MATERIALS AND METHODS

Preparation of E(gp)

E(gp) was purified from vegetative A3 and HM 2 plasma membranes by preparative gel electrophoresis. Exponential gradient SDS slab gels were prepared and loaded with 200 µg plasma membrane protein per lane as described (17). Electrophoresis was performed at 20 mA constant current at 0°C for 12 h after the entry of the tracking dye into the main separating gel. This long electrophoresis ran most proteins off the gel and left the remaining polypeptides well separated (Fig. 1). After electrophoresis, strips were cut from each edge of the gel and the remainder of the gel was frozen between glass plates to minimize breakage. The strips were stained by the PAS reaction (17) to locate the position of E(gp), the major PAS positive glycoprotein. The rest of the gel was thawed and a strip corresponding in migration to E(gp) was cut out and the contents electroeluted (24) at 6 mA for 5 h into a dialysis bag. Electroelution buffer was Laemmli SDS gel electrophoresis reservoir buffer (23). Electroelution was necessary to quantitatively remove E(gp) from the gel. If gel strips containing E(gp) were incubated overnight in 0.1% SDS, most E(gp) remained in the gel. For analytical and biological experiments, it was necessary to remove SDS and buffer from the E(gp) preparation. Therefore, the sample was dialyzed against 1,000 volumes of water for 4 days with changes of water

twice daily. During dialysis E(gp) precipitated.

Isoelectric Focusing and Lectin Labeling

Isoelectric focusing gels were prepared as previously described[1]. Gels were then fixed (22), and labeled with Con A as previously described (46) except that 2 mg fluorescein isothiocyanate conjugated Con A (FITC-Con A) was added per focusing gel. Photography of fluorescent gels was performed as usual (46) using a Type 61 Wratten filter. Isoelectric focusing gels were stained for protein with a solution of 20% 5-sulfosalicylic acid, 25% isopropyl alcohol, 0.025% Coomassie blue, and destained in 7.5% acetic acid, 5% methanol.

Amino Acid and Carbohydrate Analysis

Purified E(gp) was hydrolyzed 24, 48, or 72 h at 108°C in vacuo in 6N HCl and the resultant amino acids separated on a Durrum 500 Amino Acid Analyzer. The results were quantitated by comparison of peak heights with those from a standard mixture of amino acids. Since the protein had been in a glycine-containing buffer, a correction was made for residual free glycine by the analysis of an unhydrolyzed sample of glycoprotein.

Purified E(gp) was hydrolyzed 6 h in 4N HCl at 108°C in vacuo to determine amino sugars. The hydrolysate was then run through coupled columns of Dowex 1-X8 and 50-X4 (41) and analyzed on the Durrum 500 Amino Acid Analyzer. Analysis of carbohydrates by gas-liquid chromatography was performed as previously described[1].

Radioiodination of E(gp)

E(gp) at a concentration of 1.5 µg/ml in 1% SDS was radioiodinated by the chloramine T procedure (20).

Preparation of Lipid Vesicles

Lipid vesicles were prepared by the method of Hinkle, Kim and Racker (15). All solutions were bubbled for 30 min with N_2 before use to avoid oxidation of phospholipids. All phospholipids were purchased from Sigma Chemical Co., St. Louis, MO. In most experiments 16 µg L-α-phosphatidyl choline (PC) from bovine brain, 16 µg L-α-phosphatidyl ethanolamine (PE) from bovine brain, and about 0.75 µg E(gp) (20% of which had been radioiodinated) was dissolved in 1 ml 2% sodium cholate in 1/60 M $NaPO_4$ (pH 6.2) and dialyzed 3 x 12 h at 4°C versus 1,000 volumes of 1/60 M $NaPO_4$ (pH 6.2). Lipid vesicle preparations were then centrifuged twice for 10 min at 2500 rpm in an SS34 rotor to pellet aggregated material. In all experiments, about 25% of the labeled glycoprotein was pelleted in this step. This method produced lipid vesicles with a diameter of about 20 nm (32). Eighty percent of the E(gp) incorporated into lipid vesicles was precipitable with trichloroacetic acid as described below.

In the experiments shown in Table II, lipid vesicles were similarly prepared except that the original phospholipid and glycoprotein solutions in buffered 2% sodium cholate contained 25 µg PC, 25 µg PE and 0.38 µg E(gp) in one case and 20 µg PC, 20 µg PE,

10 µg L-α-phosphatidyl-L-serine (PS) from bovine brain, and 0.38 µg E(gp)/ml in the other case. Each preparation was dialyzed as described above.

Incubation of Cells with Lipid Vesicles

Cells were washed with 1/60 M $NaPO_4$ (pH 6.2). They were resuspended at 1 x 10^7/ml in 0.5-1.5 ml of lipid vesicle suspension prepared as described above, or mixed 1:1 with 2 x HL-5 growth medium (39) and incubated at 22°C in a 1.5 x 12 cm screw-top culture tube rotated at 20-24 revolutions/min about its long axis as described by Gerisch (10). At intervals aliquots of 100 or 200 µl were removed, diluted to 2 ml with 1/60 M $NaPO_4$ (pH 6.2) and the cells pelleted by centrifugation at 2500 rpm for 1 min in an SS34 rotor. The samples were similarly resuspended to 2 ml with 1/60 M $NaPO_4$ (pH 6.2) and pelleted twice further to wash out any residual lipid vesicles. The final cell pellet was resuspended in 2 ml 10% trichloroacetic acid and chilled at 4°C for at least 30 min. Cells were filtered onto GF/C glass fiber filters and rinsed 3 times with 4 ml 10% trichloroacetic acid. The cells were solubilized by heating the filter with 0.5 ml hyamine hydroxide at 70°C for 30 min in a sealed scintillation vial. Radioactivity was measured by liquid scintillation counting in 10 ml of fluor containing 7.9 g diphenyloxazole and 0.2 g POPOP per liter toluene.

According to the results of Papahadjopoulos et al. (35), the dilution step in sample processing described above should proportionally decrease uptake of lipid vesicles. However, in Fig. 4,

the time points in the uptake curves have been plotted 15 min after they were actually taken to include the time of processing.

We also tried to add insoluble E(gp) directly to cells. It could not be determined if this procedure was successful since insoluble E(gp) was retained by glass fiber filters in the absence of cells at such high levels that it was impossible to measure cellular uptake, if any, of radioiodinated E(gp).

Assay of Contact Sites A

At intervals, 10 µl aliquots of cells were tested for aggregation competence (formation of contact sites A) by the addition of EDTA (pH 6.0) to a final concentration of 10 mM (4).

Glutaraldehyde Fixation of Cells

Cells were glutaraldehyde-fixed by the method of Poste and Papahadjopoulos (37) for use in lipid vesicle uptake experiments and then after washing free aldehyde groups were blocked by incubation in 0.01 M glycine for 1 h at 22°C.

Indirect Immunofluorescence

Exponentially growing A3 and HM 2 cells at a concentration of 1-7 x 10^6 cells/ml or glutaraldehyde-fixed and unfixed HM 2 cells, treated for 1 h with lipid vesicles containing E(gp) from A3 cells as described above, were washed and resuspended in 0.15 N NaCl at 1 x 10^7 cells/ml. To 50 µl of cell suspension was added 50 µl unadsorbed antiserum against A3 plasma membranes or the antiserum which had been adsorbed with HM 2 cells[1]. The samples were incu-

bated 5 min on ice. 2 ml 0.15 N NaCl were added, the cells pelleted, and the supernatant decanted. The cell pellet was resuspended in 50 µl (20 mg protein/ml) fluorescein isothiocynanate-conjugated goat anti-rabbit immunoglobulin (Antibodies Incorporated, Davis, California) and incubated 5 min on ice. The cells were diluted as above, pelleted, resuspended in 0.3 ml 0.15 N NaCl, and photographed on a Zeiss fluorescence microscope. All fields of cells were photographed in both phase contrast with tungsten lamp illumination, and for fluorescence with halide arc lamp illumination and excitor filters KP490 and KP500 and chromatic reflector 510 in place.

RESULTS

Preparation and Characterization of E(gp)

E(gp) was purified by preparative SDS gel electrophoresis as described in the Materials and Methods. We decided to take advantage of the fact that glycoproteins frequently have anomalously slow migration on SDS gels (40). Therefore, SDS gels of D. discoideum plasma membranes were run 12 h instead of the usual 3.25 h to electrophorese most proteins and glycoproteins off the gel, while leaving E(gp) well separated from those which remain on the gel. Under these conditions, E(gp) ran 6.5 cm into the gel (Fig. 1). The nearest polypeptide was 0.5 cm from E(gp) on the gel (Fig. 1A). No other PAS positive glycoproteins were detectable on the gel (Fig. 1B). Therefore, a strip containing only E(gp) can easily be cut from the gel in the absence of other detectable proteins and

FIGURE 1. Preparative gel electrophoresis of E(gp).

Vegetative plasma membrane macromolecules were resolved by SDS gel electrophoresis for 12 h as described in the Methods. Lanes are:

A) HM 2 plasma membranes (50 µg protein) stained with Coomassie blue;

B) HM 2 plasma membranes (200 µg protein) stained by the PAS reaction.

The position of E(gp) is indicated.


A
B
E

glycoproteins.

Figure 2 illustrates reelectrophoresis of purified E(gp). E(gp) purified from A3 (Fig. 2, lane 3) or HM 2(Fig. 2, lane 2) plasma membranes forms a single band that comigrates with E(gp) from unfractionated plasma membranes (Fig. 2, lanes 1 and 4). The faint pair of bands at molecular weight 24,000 in lanes 2 and 3, are bleeding of polypeptides from the intense bands in the adjoining lanes of whole plasma membranes. During analytical electrophoresis (3.25 h), the migration of E(gp) is 92% of that for the standard protein β-galactosidase. Under preparative conditions (12 h electrophoresis), the migration of E(gp) falls to 67% of that for β-galactosidase. Anomalous migration has been related to the amount of SDS bound by a glycoprotein (40). Therefore, any contaminant purified along with E(gp) would have to have both similar molecular weight and anomalous migration.

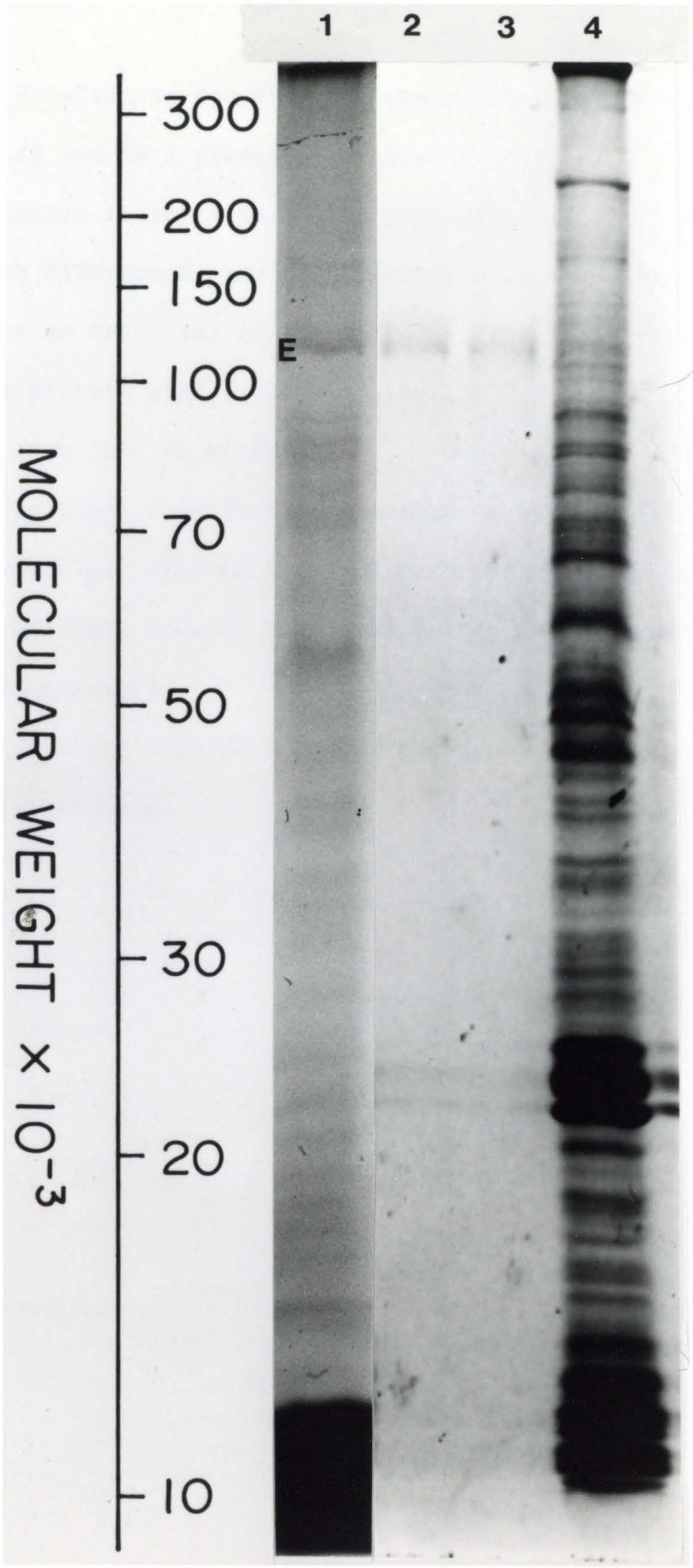
Isoelectric focusing was used to investigate the purified glycoprotein E. When the distribution of protein of E(gp) following isoelectric focusing was examined by staining with Coomassie blue, the staining of E(gp) from A3 (Fig. 3A) and HM 2 (Fig. 3B) was nearly identical in pattern and intensity. E(gp) is distributed predominantly over a pH range from 6.0-7.2 with 2 or 3 relative maxima with the bulk of the protein between pH 6.7 to 7.2. A minor amount of protein was also detected in E(gp) from HM 2 with a pI of 7.6-7.8. Most polypeptides in the plasma membrane of D. discoideum are focused into a range of less than 0.05 pH units[1]. However,

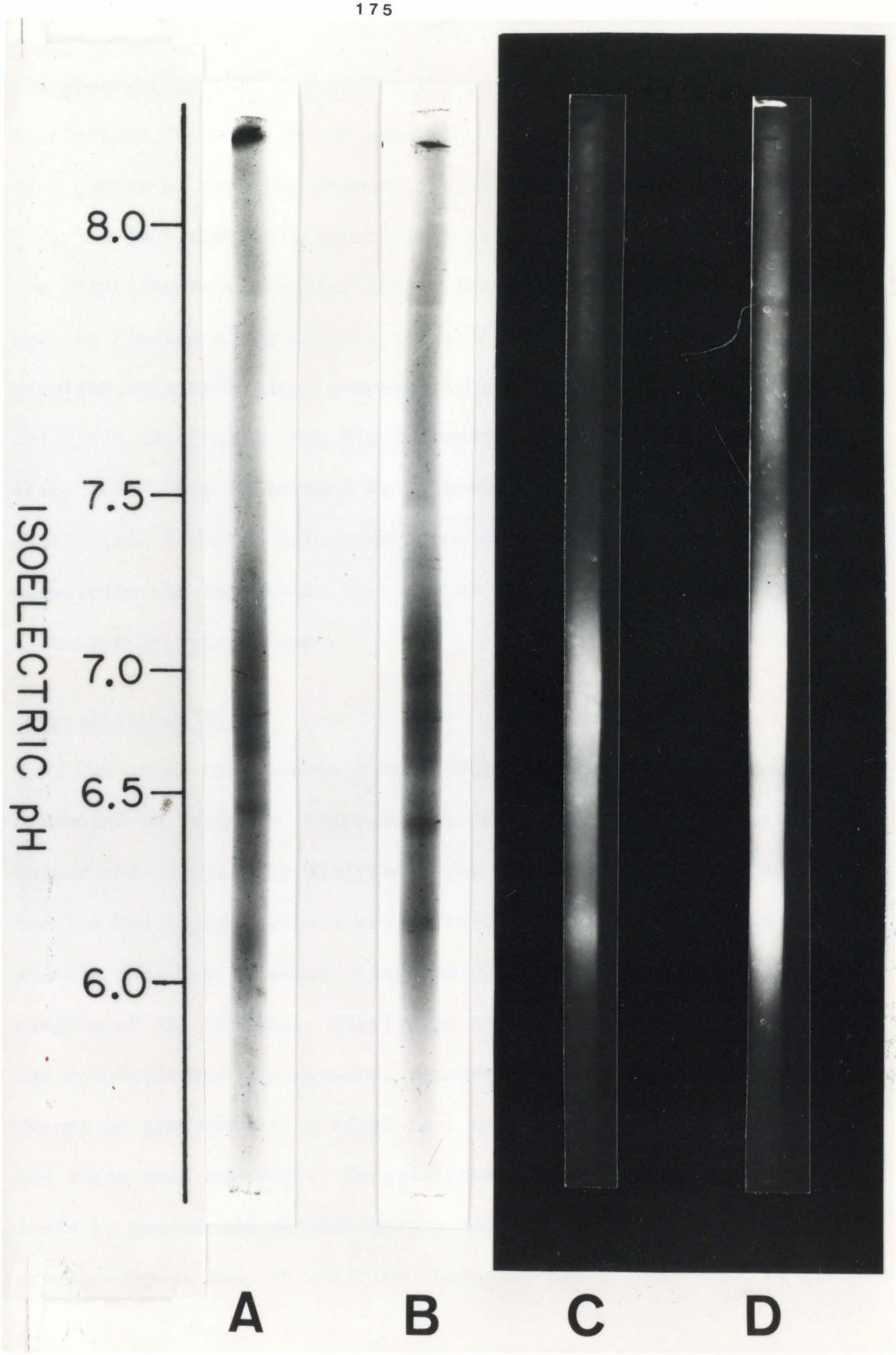
FIGURE 2. Homogeneity of isolated E(gp).

A3 vegetative plasma membranes and E(gp) isolated from A3 and HM 2 plasma membranes were analyzed by SDS gel electrophoresis. Lanes from left to right are:

1) A3 plasma membranes stained by the PAS reaction (200 µg protein content);
2) E(gp), stained for protein, purified from HM 2 plasma membranes containing 50 µg protein;
3) E(gp), stained for protein, purified from A3 plasma membranes containing 50 µg protein;
4) A3 plasma membranes stained for protein (50 µg protein content).

The letter E indicates the position of E(gp).


1
2
3
4
300
200
150
E
100
70
50
30
20
10
MOLECULAR WEIGHT × 10⁻³


ISOELECTRIC pH
8.0
7.5
7.0
6.5
6.0
A
B
C
D

the observation that a purified glycoprotein is heterogeneous upon isoelectric focusing is not unusual. Previous reports have suggested a number of possible reasons for this phenomenon (2,13,45).

The fact that this material is all E(gp) is supported by the coincidence in distribution of the pattern of protein staining and the binding of FITC-Con A (Fig. 3). In agreement with our previous results[1], E(gp) prepared from HM 2 plasma membranes (Fig. 3D) binds more Con A than E(gp) prepared from A3 plasma membranes (Fig. 3C). This difference is apparent at all positions on the isoelectric focusing gel where protein is also detectable, also supporting the contention that all of the protein is E(gp) and not a comigrating contaminant.

Composition of E(gp)

The amino acid composition of E(gp) purified from A3 cells is presented in Table I. E(gp) was prepared in a glycine-containing buffer and extensively dialyzed. The amount of glycine determined for the hydrolyzed protein was corrected for free glycine in the protein solution by subtracting the glycine content of unhydrolyzed samples of the protein. E(gp) from A3 plasma membranes contains the carbohydrates glucosamine, mannose, and perhaps glucose. The amount of glucosamine in E(gp) is 1 mole percent as determined on the amino acid analyzer. No galactosamine was detectable. Analysis by gas-liquid chromatography (GLC) also indicated the presence of glucosamine and, in addition, mannose, and a large peak of glucose.

TABLE I. Amino acid composition of E(gp).

E(gp) was isolated from A3 cells, hydrolyzed, and its amino acid composition analyzed as described in the Methods. These data are the pooled results from two preparations of A3 E(gp), one of which was hydrolyzed for 24 h, and the other for 24, 48 or 72 h. No significant differences were found between preparations or for different length hydrolyses. Therefore the data were pooled. The data are presented as mole percent $\pm$ standard deviation.

* Tryptophan was determined spectrophotometrically (7).

TABLE I

	Mole Percent
Aspartic acid	10.80 ± 0.38
Threonine	3.68 ± 0.14
Serine	10.99 ± 0.40
Glutamic Acid	15.15 ± 0.36
Proline	1.67 ± 0.15
Alanine	5.53 ± 0.22
Cysteine	0.0
Valine	5.12 ± 0.18
Methionine	1.17 ± 0.20
Isoleucine	5.64 ± 0.13
Leucine	8.11 ± 0.08
Tyrosine	2.10 ± 0.33
Phenylalanine	3.90 ± 0.10
Histidine	2.08 ± 0.13
Lysine	5.52 ± 0.08
Arginine	3.82 ± 0.12
Glycine	14.73 ± 0.33
Hydroxylysine	0.0
Hydroxyproline	0.0
Tryptophan*	<1.2

The glucose peak is believed to be predominantly due to cellulose contamination arising during dialysis. Glycoprotein samples from various sources prepared by preparative gel electrophoresis followed by electroelution into dialysis tubing routinely contain glucose contamination (J. Shively and M. Wrann, City of Hope Hospital Medical Center, Duarte, CA, personal communication). The GLC data were not quantitated because of overlap of the glucose and mannose peaks. No fucose, galactose or sialic acid was detected. Amino acid analysis indicates that 0.8% of the plasma membrane protein is E(gp) in A3 cells, and that 1.0% is E(gp) in HM 2 cells. These numbers were calculated by dividing the total mass of amino acids in an E(gp) preparation (determined by amino acid analysis) by the amount of protein in the plasma membranes from which the E(gp) was prepared as determined by the Lowry method (25).

Addition of E(gp) to Cells

The uptake of E(gp) incorporated into lipid vesicles by developing A3 cells is a multiphasic process. During the first hour of incubation, E(gp) from A3 cells (Fig. 4A) or HM 2 cells (Fig. 4B) is rapidly incorporated into developing A3 cells. Net incorporation increases at a slower rate until 6 h of incubation. The amount of incorporated E(gp) then decreases by more than 50% over the next 2.5 h. This decrease in net incorporation occurs concurrently with the attainment of aggregation competence by the A3 cells (indicated by the position of the arrow in Figs. 4A and B). The uptake kinetics of E(gp) purified from A3 cells by HM 2 cells is very

FIGURE 4. Lipid vesicle mediated incorporation of E(gp) by cells.

A3 or HM 2 cells were incubated with PC-PE lipid vesicles containing radioactive E(gp) from A3 or HM 2 plasma membranes, harvested, and counted for radioactivity as described in the Methods. The following combinations of cells and source of E(gp) are illustrated:

A) E(gp) from A3 plasma membranes incubated with A3 cells in phosphate buffer;

B) E(gp) from HM 2 plasma membranes incubated with A3 cells in phosphate buffer;

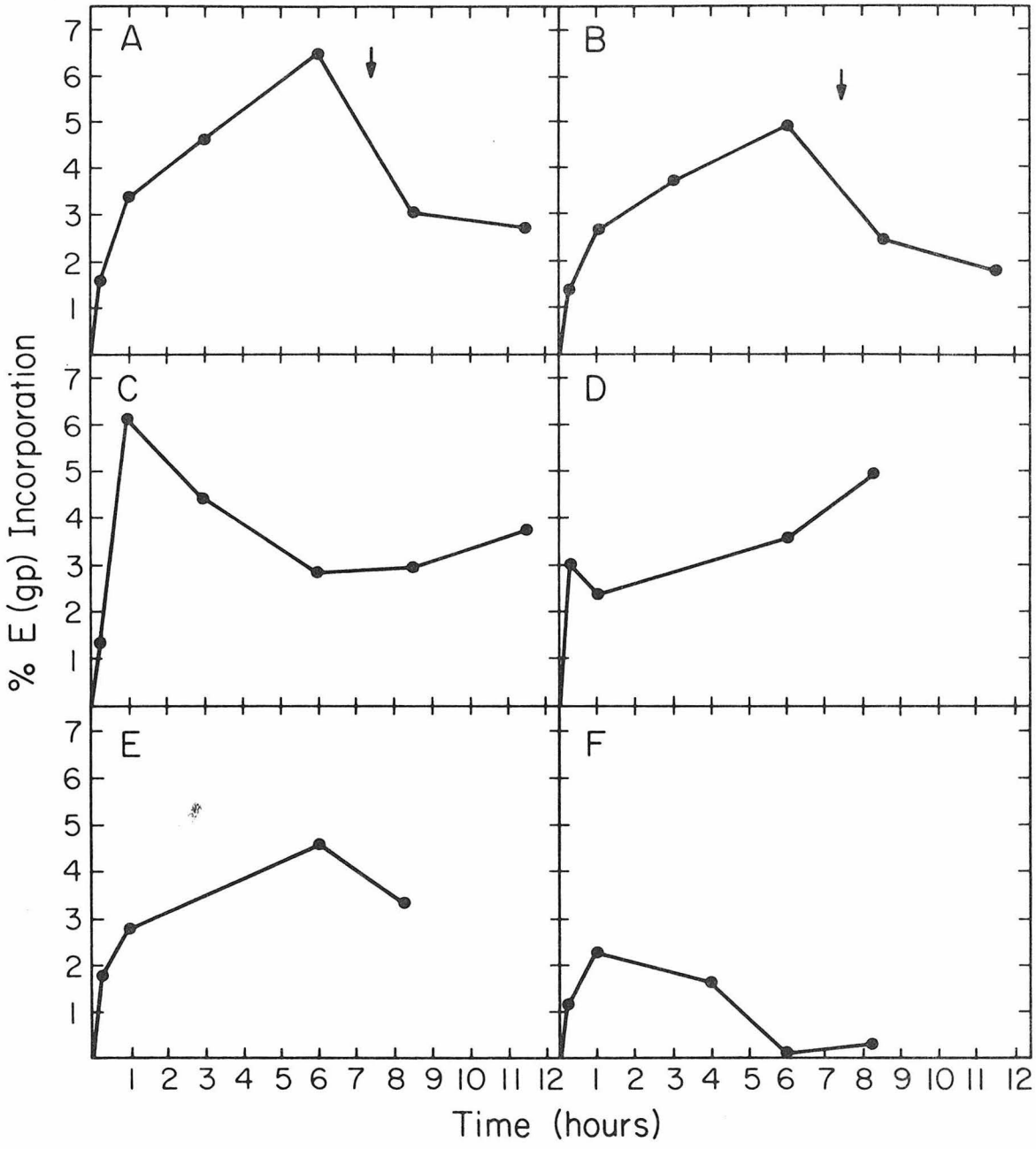
C) E(gp) from A3 plasma membranes incubated with HM 2 cells in phosphate buffer;

D) E(gp) from A3 plasma membranes incubated with A3 cells in HL-5 nutrient medium;

E) E(gp) from A3 plasma membranes incubated with glutaraldehyde-fixed A3 cells in phosphate buffer;

F) E(gp) from A3 plasma membranes incubated with A3 cells (incubation buffer supplemented with 500 μg/ml cycloheximide).

The arrows in panels A and B indicate the point at which greater than 90% of the cells had attained full aggregation competence.


A
B
C
D
E
F
% E (gp) Incorporation
Time (hours)
1 2 3 4 5 6 7 8 9 10 11 12
1 2 3 4 5 6 7

different. Maximal incorporation is observed in HM 2 cells at 1 h (Fig. 4C). Thereafter total incorporation of E(gp) decreases for several hours before leveling off. Therefore, HM 2 cells show a net loss of added E(gp) for several hours during which A3 cells are still showing a net incorporation of the glycoprotein. Similar results were obtained when E(gp) from HM 2 cells was incubated with HM 2 cells (data not presented). Vegetative A3 cells in growth medium also incorporate E(gp) (Fig. 4D) and a decrease in net incorporation of E(gp) was not observed during the course of this experiment.

A3 cells were prefixed with glutaraldehyde (Fig. 4E) or treated with cycloheximide (Fig. 4F) to study the effect of these inhibitors on E(gp) incorporation. Glutaraldehyde-fixed A3 cells incorporate E(gp) at least 70% as well as unfixed cells (Fig. 4E). Glutaraldehyde-fixed HM 2 cells incorporate E(gp) with similar kinetics and extent of maximal incorporation (data not shown). The uptake of lipid vesicles or their soluble contents by glutaraldehyde-fixed cells have been interpreted by other workers as due to fusion of vesicles with cells as the primary means of vesicle incorporation (3,19,37).

Cycloheximide treated A3 cells incorporate E(gp) at an almost normal rate for 1 h, but then appear to cease incorporating the glycoprotein and to lose previously incorporated E(gp) (Fig. 4F). The effects of cycloheximide on E(gp) incorporation could be due to any one of the many effects cycloheximide treatment has on cells

(29). But it is interesting that cycloheximide causes the premature loss of many external membrane proteins (but not E(gp)) in developing D. discoideum cells[2].

Ca^{++} (4 mM) increases the extent of E(gp) incorporation by A3 cells (Table II). This result might be expected for PS-containing vesicles since Ca^{++} has been reported to induce fusion among such vesicles (32,36). However, the uptake of E(gp) from vesicles containing only neutral phospholipids is also slightly stimulated by Ca^{++}.

Indirect Immunofluorescence

Antiserum, made against A3 plasma membranes and adsorbed with HM 2 cells, bound to A3 cells but not to HM 2 cells. Previous experiments demonstrated that this antiserum recognized two plasma membrane molecules on A3 cells[1], E(gp) and a molecule of molecular weight 150,000. The 150,000 dalton molecule is not expressed on HM 2 cells and E(gp) has altered antigenicity[1]. Therefore, it appeared possible to demonstrate the association of A3 E(gp) originally in lipid vesicles into the HM 2 plasma membrane. This added E(gp) would be the only molecule present recognized by the HM 2-adsorbed antiserum.

In Figures 5 and 6 each pair of pictures (A-B, C-D, etc.) show the same field of cells photographed in phase contrast (left) or by fluorescence (right). Unadsorbed antiserum against A3 plasma membranes binds extensively to A3 (Fig. 5A,B) or HM 2 (Fig. 5C,D) cells. Following adsorption of this serum by HM 2 cells, A3 cells

TABLE II

Ca^{++} STIMULATION OF E(GP) INCORPORATION

Lipid Composition of Vesicles	% E(gp) Incorporation		Ca^{++} Stimulation (%)
	$-Ca^{++}$	$+Ca^{++}$	
PC-PE (1:1)	5.6	7.3	30.4
PC-PE-PS (2:2:1)	3.7	7.7	108.1

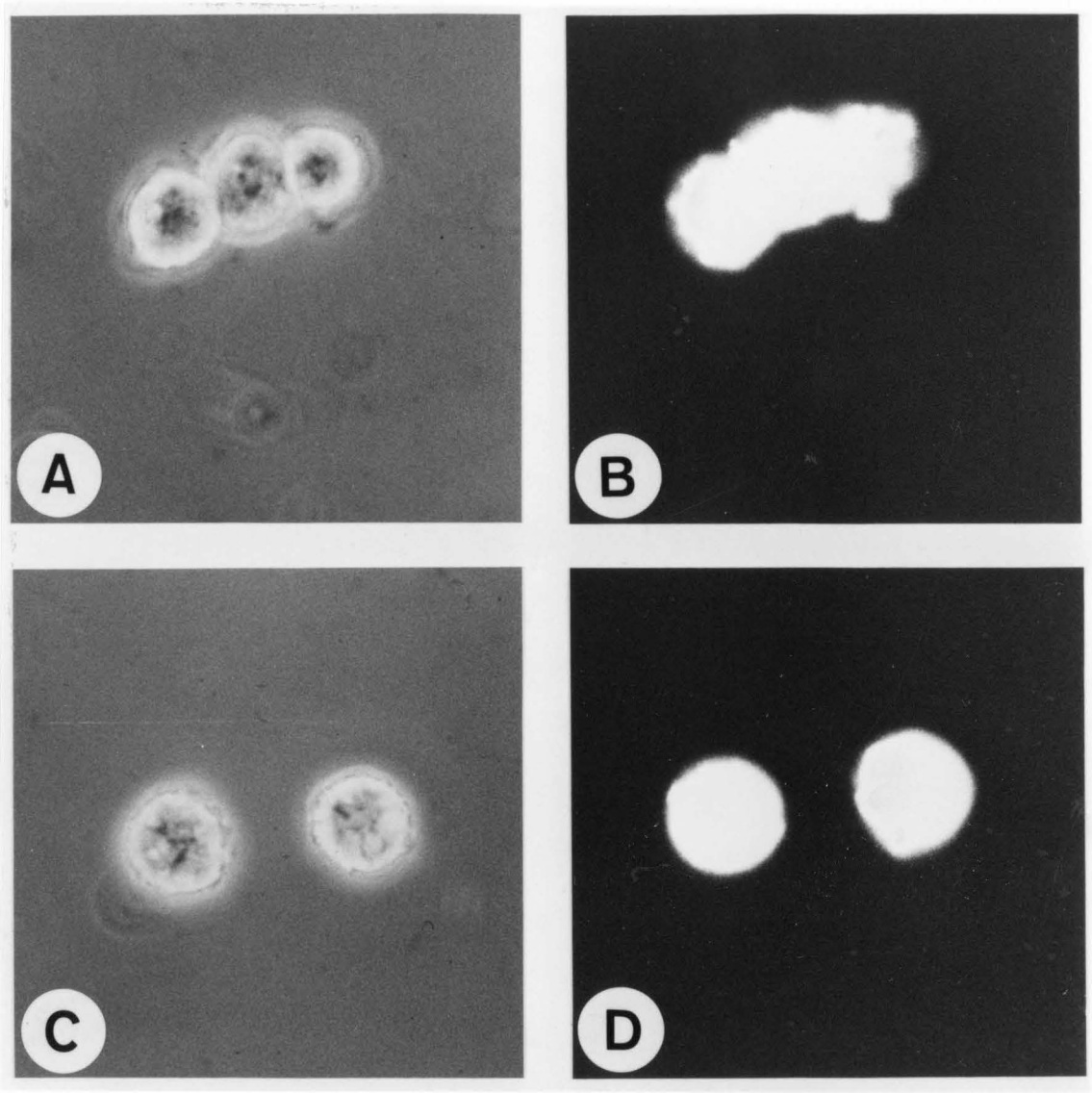
Lipid vesicles containing E(gp) from A3 cells and the indicated lipids were incubated with A3 cells for 3 h plus or minus 4 mM $CaCl_2$, harvested and radioactivity counted as described in the Materials and Methods.

FIGURE 5. Labeling of A3 and HM 2 cells with antibody to plasma membranes.

A3 and HM 2 cells were sequentially reacted with unadsorbed rabbit antiserum against A3 plasma membranes and then FITC-goat anti-rabbit antibody as described in the Methods. Identical fields of cells (A-B, C-D) were photographed in phase contrast (A,C) or by fluorescence (B,D).

A-B) A3 cells reacted with unadsorbed serum;

C-D) HM 2 cells reacted with unadsorbed serum.


A
B
C
D

are still lightly labeled (Fig. 6A,B), but HM 2 cells are unlabeled (Fig. 6C,D). However, the A3 specific antigens are not expressed on all A3 cells (Fig. 6A,B). We estimated the fraction of A3 cells which were labeled by locating cells with phase contrast and then photographing the fluorescence. Fluorescent cells were scored on photographs in order to eliminate the possibility that fluorescence was being bleached during observation and thus yielding only the appearance of unlabeled cells. In total, 9 of 26 A3 cells, photographed and scored, were not labeled by this antiserum. The absence of bound antibody is not likely to be due to the shedding or endocytosis of capped antibody since such a process requires about 60 min at room temperature (33), while these experiments were done at $0^{o}C$.

After living HM 2 cells were treated with lipid vesicles containing E(gp) from A3 cells, they bound HM 2 adsorbed antiserum. The incorporated E(gp) is found in a relatively patchy distribution (Fig. 6E,F,G, and H).

We also examined the incorporation of exogenous E(gp) in lipid vesicles by glutaraldehyde-fixed HM 2 cells. Glutaraldehyde-fixed HM 2 cells are slightly autofluorescent (Fig. 6I,J). When fixed HM 2 cells were treated only with the FITC-goat anti-rabbit immunoglobin, they gave identical results (data not shown). However, fixed HM 2 cells treated with lipid vesicles containing E(gp) from A3 cells label with adsorbed antiserum (Fig. 6K,L). These results support the belief that exogenously added E(gp) in lipid vesicles

FIGURE 6. Labeling of A3 and HM 2 cells with adsorbed antibody.

A3 cells, HM 2 cells treated with lipid vesicles containing E(gp) from A3 cells for 1 h, or untreated HM 2 cells were sequentially reacted with HM 2-adsorbed rabbit antiserum against A3 plasma membranes and fluorescent goat anti-rabbit antibody as described in the Methods. Identical fields of cells (A-B, C-D, E-F, G-H, I-J, K-L) were photographed in phase contrast (A,C,E,G,I,K) or by fluorescence (B,D,F,H,J,L).

A-B) A3 cells;

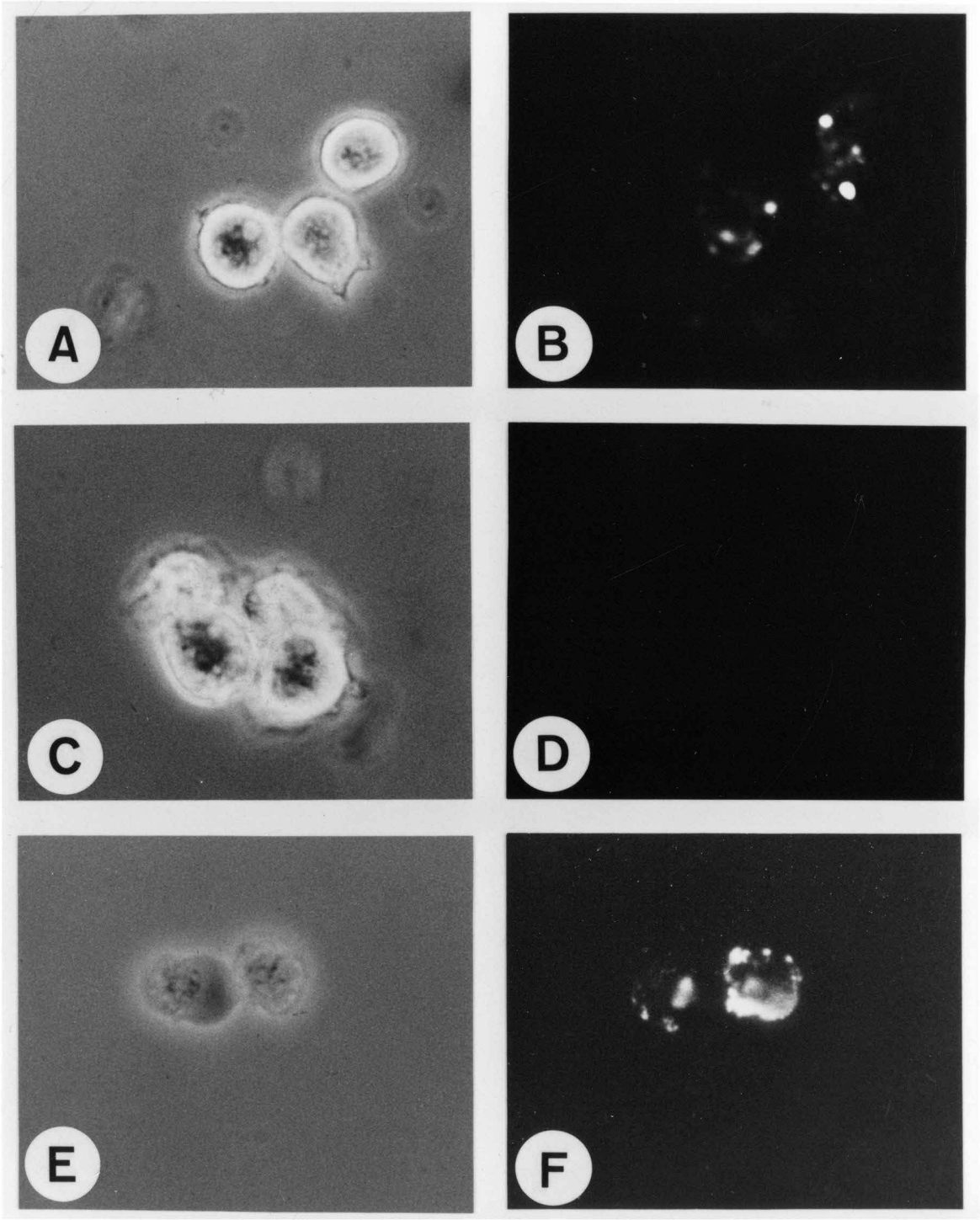
C-D) HM 2 cells;

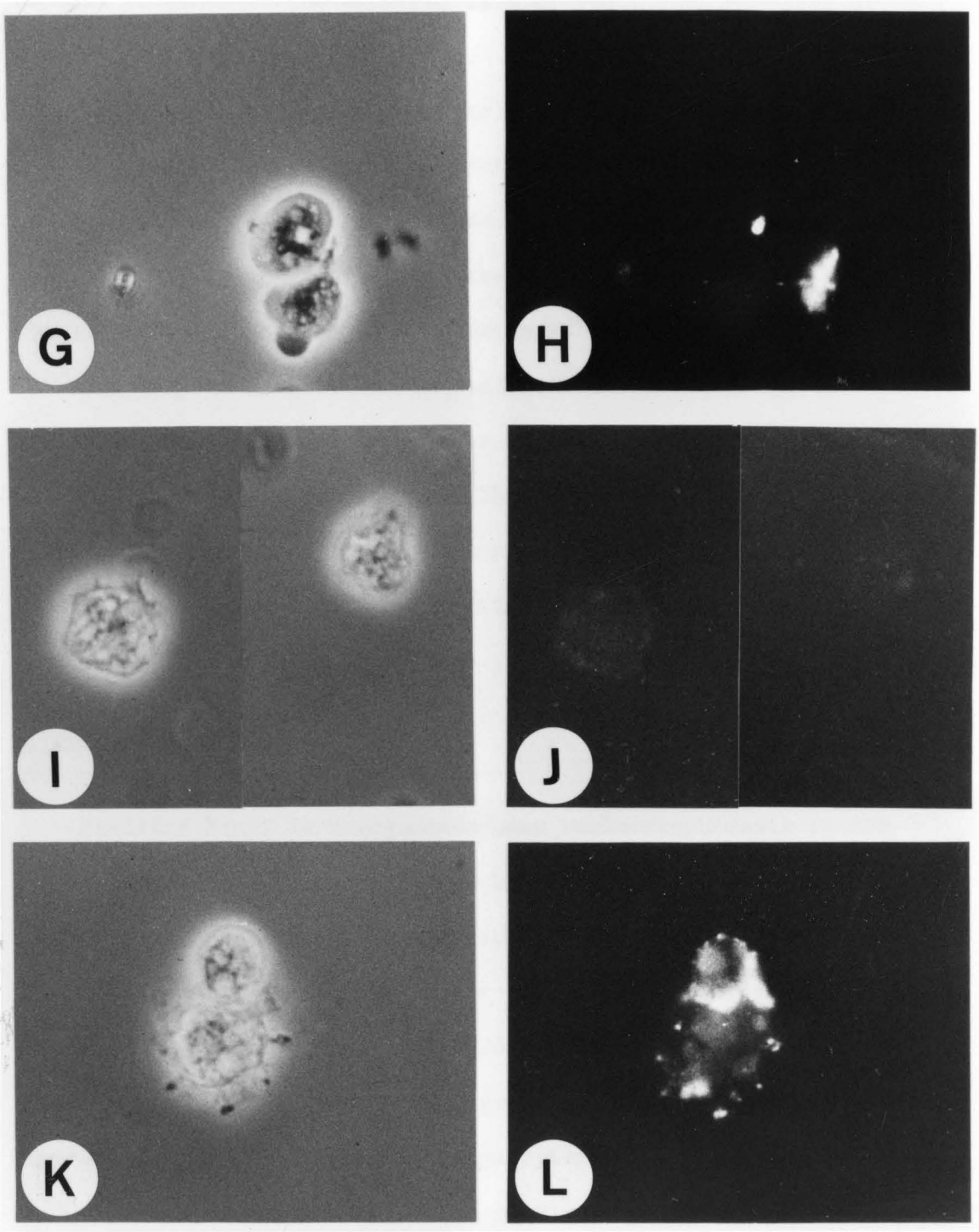
E-F) HM 2 cells treated with lipid vesicles containing E(gp) from A3 cells;

G-H) HM 2 cells treated with lipid vesicles containing E(gp) from A3 cells;

I-J) Glutaraldehyde-fixed HM 2 cells (not treated with lipid vesicles);

K-L) Glutaraldehyde-fixed HM 2 cells treated with lipid vesicles containing E(gp) from A3 cells.


A
B
C
D
E
F


G
H
I
J
K
L

becomes attached to the external face of the plasma membrane of treated cells.

Lipid vesicles containing E(gp) were incubated with cells and the time of formation of contact sites A was assayed as an indicator of aggregation competence. Lipid vesicles made without E(gp) or containing E(gp) from either A3 or HM 2 cells all slowed the attainment of aggregation competence by A3 cells 1 h (to 7.5 h incubation) as compared to untreated A3 cells which aggregated in 6.5 h. None of these treatments allowed the development of HM 2 cells to aggregation competence. Therefore, E(gp) from A3 or HM 2 cells can be reassociated with the plasma membrane of wild type D. discoideum cells without disrupting development to aggregation competence.

DISCUSSION

Purified E(gp) is homogeneous upon reelectrophoresis on SDS gels. Although E(gp) was purified by preparative gel electrophoresis, it is unlikely to contain comigrating contaminants since it has a different apparent molecular weight and formed a single, discrete band during electrophoresis for 3.25 h (Fig. 2) or 12 h (Fig. 1) on SDS gels made with an exponential gradient of polyacrylamide. Due to the exponential gradient of polyacrylamide in our gels, the two different times of electrophoreses of E(gp) are equivalent to electrophoresis on gels of different acrylamide concentration. Any contaminant to E(gp) would have to comigrate with E(gp) under both

different electrophoretic conditions and thus show the same anomalous migration as compared to standard proteins. Glycoproteins have been shown to have anomalous behavior when electrophoresed through differing concentrations of polyacrylamide (40). Thus a change in acrylamide concentration from 5 to 12.5% leads to a 60% change in the apparent molecular weight of glycophorin (40). This aberrant behavior has been ascribed to differences in the ratio of bound SDS to mass of the molecule between proteins and glycoproteins and among glycoproteins (40). The change in the mobility of E(gp) relative to β-galactosidase shows that it too has anomalous migration. The facts that all of the purified material bound concanavalin A and that E(gp) from HM 2 bound uniformly more concanavalin A support the apparent homogeneity of the glycoprotein. However, the reason for its broad distribution on isoelectric focusing, even though normal for glycoproteins (2,13,45), must be investigated before we can be completely confident of the homogeneity of purified E(gp).

No striking similarities were found between the amino acid composition of E(gp) and the amino acid compositions of other membrane proteins including those of the major erythrocyte plasma membrane glycoproteins glycophorin (43) and band 3 (21). E(gp) has a relatively high percentage of polar amino acid residues (polarity index as defined by Capaldi and Vanderkooi in reference 6). Fifty-two percent of the amino acid residues in E(gp) from A3

cells are polar. Only 1 of 19 membrane proteins examined by Capaldi and Vanderkooi (calsequestrin), had a higher polarity index, 53.6%. Since that study, amino acid analyses for band 3 (21) and glycophorin (43) have been published. Our calculation indicates that band 3 has a polarity index of 43.3%, and that glycophorin (which is water soluble following purification) has a polarity index of 54.6%, even higher than that of E(gp). The high polarity of E(gp) might suggest that extensive regions of this molecule are normally in contact with an aqueous environment. No other classes of amino acids were present in unusual amounts. The presence of large amounts of aspartic acid, serine and threonine are consistent with the potential to carry many carbohydrate chains since asparagine, serine and threonine are the major unmodified amino acids involved in carbohydrate-peptide linkages (42). The amino acid composition of E(gp) indicates a minimum possible molecular weight of about 18,000 daltons for its polypeptide portion. This estimate was arrived at by assuming that the least common amino acids in A3 E(gp); methionine 1.17%, proline 1.67%, histidine 2.08%, and tyrosine 2.10%, respectively are present in 2,3,4 and 4 residues per molecule of E(gp).

E(gp) contains about 1 mole percent glucosamine as determined on the amino acid analyzer. E(gp) does not bind detectible WGA[1] suggesting that residues of N-acetylglucosamine in this molecule are substituted or are dispersed in the carbohydrate chains so that polymers of N-acetylglucosamine, such as N-acetylchitobiose (which

has a 600-fold higher affinity for WGA than N-acetylglucosamine(1)) are not present. The presence of mannose, and a very large peak of glucose in our E(gp) preparations was determined by GLC separation of the trifluoroacetyl derivatives of the methyl glycosides followed by detection by electron capture. The relative amount of mannose present could not be accurately quantitated because of the large overlapping glucose peak and because of the small amount of E(gp) available for this analysis. It is possible that E(gp) does contain some glucose since D. discoideum cells synthesize extracellular glucose-containing macromolecules such as glycogen, cellulose, and a secreted glycoprotein (48). However, this must remain tentative because of the possibility of glucose contamination as discussed in the Results. Carbohydrate chains containing mannose and/or glucose are consistent with the ability of E(gp) to bind Con A.

Indirect immunofluorescence experiments with HM 2 adsorbed antiserum against A3 plasma membranes indicated that A3 cells have cell surface antigens not found on HM 2 cells[1]. However, these antigens are not uniformly expressed on all A3 cells (Fig. 6A,B). Previous work indicated these antigens are associated with two macromolecules, E(gp) and a 150,000 dalton macromolecule[1]. Therefore, the cell surface exposure of both these molecules apparently differs among a population of A3 cells. Approximately one-third of the vegetative cells examined did not show these antigens. Exposure of receptors on the cell surface has been shown to change during the cell cycle in other systems (8,44) so that it is possible that a similar phenomenon occurs for E(gp) and the 150,000 dalton

macromolecule in D. discoideum. Previous observations (17) showed that polypeptides in the plasma membrane of D. discoideum change in their surface exposure during development.

When the incorporation of exogenous E(gp) in lipid vesicles by developing cells was monitored by following the uptake of radioiodinated glycoprotein, the kinetics and magnitude of E(gp) incorporation in A3 cells were initially similar to that observed (on a per cell basis) by Batzri and Korn (3) for the incorporation of phospholipid vesicles by the related soil amoeba, Acanthamoeba castelanii. The kinetics were also similar to those observed for the incorporation of vesicles by cultured mammalian cells (19,35) but the magnitude of uptake cannot be directly compared because the mammalian cells were in monolayer culture and are not uniformly exposed to the vesicles. The maximum uptake of E(gp) is equal to the incorporation of an additional 45% of the amount of E(gp) normally present in vegetative cells.

Concurrent with the time of aggregation, A3 cells lose 50% of previously incorporated E(gp). HM 2 cells also lose 50% of previously incorporated E(gp) but do so earlier during incubation. The difference in the time course of exogenous E(gp) incorporation by A3 and HM 2 cells indicates that the relative rates of incorporation and/or turnover are different in the two strains. Continuing incorporation of E(gp) should be possible throughout these experiments since even after 6 h incubation with cells, 46% of the original E(gp) label is maintained in the supernatant and can be

precipitated by trichloroacetic acid. The relative importance of decreases in uptake and of degradation and/or release of the incorporated glycoprotein can, however, only be determined by pulse-labeling the cells with E(gp) at various times during development. The decrease in net exogenous E(gp) incorporation in A3 and HM 2 cells is qualitatively similar to the partial loss of endogenous E(gp) observed by PAS staining of SDS gels of plasma membranes from A3 and HM 2 cells which have been incubated for development[2] (17).

It is interesting that E(gp) continued to show net incorporation in vegetative cells throughout the experiment. Such a result is in accord with the maintenance of endogenous E(gp) by vegetative cells. However, cycloheximide treatment also maintains endogenous E(gp) in cells incubated for development[2] (16), but it causes the loss of exogenously added E(gp). It should be noted that cycloheximide also causes the premature disappearance of a number of external polypeptides in D. discoideum.[2]

Three experiments indicate that the incorporation of E(gp) is not mediated mainly by pinocytosis or phagocytosis. Ca^{++} promotes the incorporation of E(gp) into cells (Table II), but has been shown to inhibit pinocytosis of sucrose, lysozyme, and ribonuclease in the amoeba, Chaos chaos (5). Stronger evidence is provided by the incorporation of vesicles into glutaraldehyde-fixed target cells. This has been taken as evidence by many groups for vesicle fusion with the plasma membrane and against endocytosis of lipid vesicles (3,19,37). Lastly, indirect immunofluorescence

gives direct proof for an association of exogenous E(gp) with the plasma membrane of target cells.

E(gp) might be associated with the membrane either by vesicle fusion or by direct transfer from vesicles to cells. Pagano and Huang (34) provided some evidence for the occurrence of direct exchange of lipids from cells to vesicles. Presumably treatment with Ca^{++} or glutaraldehyde fixation would not prevent a similar direct transfer of E(gp) from vesicles to cells.

This study indicates the display of a macromolecule on the surface of the plasma membrane of D. discoideum cells can be altered by incubation of the cells with lipid vesicles containing the macromolecule. This opens an avenue for a wide variety of experiments.

ACKNOWLEDGMENTS

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FOOTNOTES

[1]Hoffman, S., and McMahon, D. (1977) Defective Glycoproteins in the Plasma Membrane of an Aggregation Minus Mutant of Dictyostelium discoideum with Abnormal Cellular Interactions, submitted for publication.

[2]Hoffman, S., and McMahon, D. (1977) The Effects of Inhibition of Development on Changes in Plasma Membrane Composition and Topography, submitted for publication.