PROTEASE SECRETION DURING ONSET OF DEVELOPMENT IN *DICTYOSTELIUM DISCOIDEUM*

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SUMMARY

At the onset of development, the single cells of the eukaryotic micro-organism *Dictyostelium discoideum* secrete proteolytic activity which can be assayed using the insoluble substrate remazolbrilliant blue hide. The activity is not secreted by exponentially growing cells, but does appear extracellularly at the onset of the stationary growth phase. When growth phase cells are resuspended in non-nutrient buffer, proteolytic activity begins to appear outside the cells. It accumulates in the buffer at a rate similar to that observed for 2 glycosidases of lysosomal origin and reaches a maximum after about 2 h of incubation. After 3–4 h incubation, centrifugation of the non-nutrient buffer removes the cells, producing a supernatant which we refer to as conditioned medium.

Subsequent experiments with conditioned medium showed: (a) its incubation with purified plasma membranes results in the release of polypeptides which can be recovered and, when displayed on polyacrylamide gels, can be shown to be stage specific; and (b) that conditioned medium can decrease the rate of detachment of cells from a collagen substratum. Both effects can be prevented by the addition of remazolbrilliant blue hide suggesting that they are due to proteolytic activity present in the conditioned medium.

Finally, we were able to show that conditioned medium contains components which, when spread over the bottom of plastic Petri dishes, enhance the rate of multicellular structure formation. Additional studies showed that this effect of conditioned medium could also be brought about by components which remained behind on uncoated plastic dishes after the removal of a *D. discoideum* cell layer. These data may be accommodated to a model in which the protease secreted during the onset of development acts on the cell membrane releasing components which coat the substratum and facilitate migration and multicellular structure formation.

INTRODUCTION

After growth, the single cells of the eukaryotic micro-organism *Dictyostelium discoideum* aggregate and construct fruiting bodies. The overall process of aggregation can be subdivided into 2 events: the migration of the individual cells toward central collecting points, and the formation of an organized multicellular structure. While the process of migration has been correlated with chemotaxis and the formation of multicellular structures has been correlated with both morphological (Aldrich & Gregg, 1973; Rossomando, Steffek, Mujwid & Alexander, 1974) and biochemical changes at the cell surface (Smart & Hynes, 1974; Geltosky, Siu & Lerner, 1976; Beug, Katz & Gerisch, 1973; Malchow & Gerisch, 1974; Malchow, Nagele, Schwartz
& Gerisch, 1972; Rosen, Kafka, Simpson & Baronde, 1973; Siu, Lerner, Ma, Firtel & Loomis, 1976), the possibility that processes occur which mediate a transition between migration and multicellular structure formation remains to be considered.

Recent studies suggest that proteases may be involved in these processes. For example, based on studies using chick embryos, it has been suggested that alterations of cell surface components by proteolytic activities may promote an increase in intercellular adhesion (Rutishauser, Thiery, Brackenbury, Sela & Edelman, 1976). Since the cells of D. discoideum which are in a process of transition from migration to multicellularity must also change their adhesive properties, we have in the present work explored the role of proteolytic activity in the overall aggregation process. We have used the insoluble protein remazolbrilliant blue hide as substrate (Rinderknecht, Geokas, Silverman & Haverback, 1968) since Dancer & Mandelstam (1975) have pointed out that this reagent is more sensitive to low levels of proteolytic activities than other protein-based substrates. Using this substrate we have been able to detect proteolytic activity in the media of starving cells. In addition to studies on the kinetics of its release, we have examined the effect of proteolytic activity on isolated plasma membranes. Finally, we have studied the effect of proteolytic activity on the cell-substratum interaction using a new method for measuring the rate of detachment of the cells from collagen-coated plastic dishes. This procedure is based on observations made in the laboratory which showed that cells in different stages of growth and development vary in their rates of detachment from this substratum. With this assay, we have been able to study directly the role of proteolytic activity in modifying this property of the cells.

EXPERIMENTAL PROCEDURES

Materials

Remazolbrilliant blue hide (Hide Powder Azure, B grade) was obtained from Calbiochem. Acrylamide, bisacrylamide and $N,N',N'',N'''$-tetramethylethylenediamine (TEMED) were obtained from Eastman. Triton X-100 was from Schwarz/Mann. Phenylmethylsulphonylfluoride (PMSF), 1,10-phenanthroline, tosyl-L-lysine chloromethyl ketone (TLCK), and $\beta$-nitrophenyl glycosides were from Sigma.

Organisms and conditions for growth

The Ax-3 strain of Dictyostelium discoideum derived from the parent stock, NC-4, was used throughout. Cells were grown in HL-5 medium on a gyratory shaker as previously described (Rossomando et al. 1974). Under these conditions, the cells have a doubling time of about 9-10 h.

Preparation of aggregation-competent cells

Cells were harvested from exponential growth phase ($1-2 \times 10^6$ cells/ml) and washed twice with $0.015 \ M \ \text{KH}_2\text{PO}_4$ (pH 6.1), 2 mM MgSO$_4$ (KPM buffer) and resuspended in the same buffer at $1 \times 10^7$ cells/ml and incubated, with shaking, at 22 °C for 6 h. The time of transfer into the buffer is taken as time zero.
Preparation of conditioned media

Cells were harvested from KPM 4 h after the onset of starvation by centrifugation at 5000 g for 5 min. The supernatant (conditioned media) was dialysed overnight at 4 °C against 100 vol. of deionized water, and either used in this form or it was lyophilized and stored at −20 °C until needed.

Protease assay conditions

Proteolytic activity was measured by following the solubilization of the insoluble substrate remazolbrilliant blue hide (RBBH) using a procedure based on one described by Dancer & Mandelstam (1975) modified as follows. One millilitre of the sample to be tested was incubated with 20 mg RBBH, 0.1 ml 10 % (v/v) Triton X-100, 0.1 ml 0.1 M sodium acetate (pH 4.0), and 3.8 ml KPM buffer for 30 min at 30 °C. The reaction was terminated by passing the reaction mixture through a filter (Whatman No. 42). The extent of RBBH solubilization was determined from the optical activity at 595 nm of the filtrate. One unit of activity is defined as that amount of enzyme which will change the optical density at 595 nm by 0.1 unit in 30 min at 30 °C.

Glycosidase assay conditions

β-N-acetylglucosaminidase and α-mannosidase activities were determined essentially as described by Every & Ashworth (1973). Enzyme preparations were incubated at 30 °C for 10–60 min in 0.05 M sodium acetate buffer (pH 4.5) containing 5 mM p-nitrophenyl glycoside. Reactions were stopped by the addition of 9 vol. of 1 M Tris-HCl (pH 9.0) and the absorbance was measured at 400 nm.

Isolation and purification of plasma membranes

Cells were grown to the required stage, harvested and lysed using amphotericin B and plasma membranes isolated by differential centrifugation and purified on a discontinuous sucrose gradient as previously described (Rossomando & Cutler, 1975).

Incubation of plasma membranes with conditioned media

Plasma membranes were resuspended in 1 ml of 0.1 M Tris-HCl (pH 7.5) buffer at a final concentration of 20 mg/ml. Approximately 0.4 ml of membranes was incubated together with 0.2 ml (approx. 0.7 mg protein/ml) of conditioned media, and the mixture incubated at 30 °C for 18 h. The incubation was terminated by centrifugation (30,000 g, 15 min) and the supernatant recovered and prepared for electrophoresis as described below. For control incubations, remazolbrilliant blue hide was added to the incubation mixture at a final concentration of 20 mg/ml.

Sodium dodecylsulphate-polyacrylamide gel electrophoresis

Electrophoresis was carried out in a sodium dodecylsulphate-polyacrylamide system with a 3 % acrylamide stacking gel (2 cm) and a 7.5 % separating gel (8 cm). Samples were run in a buffer containing 25 mM Tris, 192 mM glycine, and 0.1 % sodium dodecylsulphate (pH 8.0). Samples were prepared in a solubilizer containing 25 mM Tris, 192 mM glycine (pH 8.0), 1 % sodium dodecylsulphate, and 1 % 2-mercaptoethanol at an approximate protein concentration of 1 mg/ml. Complete solubilization was assured by heating samples at 100 °C for 2 min immediately before application to gels. Five μl of 0.1 % bromphenol blue were added to each sample as tracking dye. A few crystals of sucrose were also added to each sample to increase density. Electrophoresis was carried out on a Hoeffer apparatus at a constant current of 2.5 mA/gel. Running time under these conditions was no more than 4 h. Following electrophoresis, gels were stained for protein with Coomassie Blue. Stained gels were scanned at 550 nm on a Gilford Model 2400-S spectrophotometer with a Model 2410 linear transport accessory.
Cell-substratum detachment assay using collagen-coated plastic dishes

For this assay plastic Petri dishes (Falcon 60 x 15 mm–no. 1007) were coated with reconstituted rat-tail collagen (Bornstein, 1958). Approximately 0.4 ml (4 x 10^6 cells/ml) of cells harvested from appropriate stages were spread on the plate and, at intervals, the supernatant was poured off and the plate washed with 0.4 ml 0.1 M Tris-HCl, pH 7.5. Following gentle shaking, this buffer was poured off and the titre of the cells recovered in the buffer determined by counting in a haemocytometer. No additional cells could be removed by subsequent buffer washes.

RESULTS

Growth-dependent changes in extracellular proteolytic activity

Using the remazolbrilliant blue hide (RBBH) as substrate, proteolytic activity was assayed in the extracellular medium (HL-5) at 2 representative stages of growth of the *D. discoideum*. For these experiments, after the required stage of growth had been reached, the cells were removed by centrifugation, the supernatant dialysed and an aliquot assayed for proteolytic activity. The activity levels obtained are shown in Table 1. While exponentially growing cells contain barely detectable levels of activity, the activity reaches a significant level after the cells reach stationary phase.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Activity (units*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exponential growth (1–2 x 10^6 cells/ml)</td>
<td>0.002</td>
</tr>
<tr>
<td>Stationary phase (1–2 x 10^7 cells/ml)</td>
<td>0.356</td>
</tr>
</tbody>
</table>

* Cells harvested at appropriate stage of growth and supernatant recovered and dialysed against 100 vol. deionized water overnight at 4 °C. Protease activity assayed as described. In control experiments 0.150 unit of proteolytic activity was added to dialysed medium and approximately 95% could be detected. One unit of activity is that amount of enzyme which will change the optical density at 595 by 0.1 unit in 30 min at 30 °C.

To determine if the low levels of proteolytic activity at the exponential growth phase were due to the presence of an inhibitor in the growth phase media, 0.15 units of dialysed and lyophilized proteolytic activity obtained from conditioned medium (see Experimental procedures) was added to the growth medium obtained from growing cells. When this reconstituted medium was assayed, approximately 95% of the proteolytic activity could be detected.

Kinetics of appearance of proteolytic activity in non-nutrient buffer

The single amoeboid cells of *D. discoideum* become aggregation competent following incubation in a non-nutrient medium (KPM) after about 6 h of incubation (Lee, 1972). The results of our initial studies showed that, following removal of cells from KPM after a 4-h incubation, proteolytic activity could be detected in this cell-free buffer (conditioned medium). The time-course of the appearance of this proteolytic activity in the conditioned medium (CM) is shown in Fig. 1. Activity begins to appear immediately after resuspending the cells in KPM and reaches its maximum after about 2–3 h.
For purposes of comparison, we determined the rate of release of 2 lysosomal glycosidases, previously shown to be secreted when the growth of *D. discoideum* ceased (Weiner & Ashworth, 1970). The time course of the appearance of these 2 activities in the CM is also shown in Fig. 1. A comparison of the results clearly shows that the rates are similar.

![Graph showing time course of release of enzymic activities from cells following onset of starvation.

Fig. 1. Time course of release of enzymic activities from cells following onset of starvation. Cells harvested from growth phase (1-2 x 10^6 cells/ml) were resuspended in 25 ml 0.015 M KH_2PO_4 (pH 6.1) containing 2 mM MgSO_4 (KPM buffer) at a final concentration of 1 x 10^7 cells/ml and incubated with shaking at 22 °C. At intervals the cells were removed from the solution by centrifugation at 5000 g for 10 min and the supernant recovered and stored at -80 °C. Prior to enzyme analysis, Triton X-100 was added to the supernant to a final concentration of 1 % and approximately 1-5 ml (200 /μg protein) were assayed. Protease (△—△) and glycosidase (□—□) activities were assayed as described in Methods. Activities given as percent of maximum value obtained after 4 h incubation in KPM.

In order to characterize the proteolytic activity, the CM was dialysed overnight at 4 °C against 100 vol. of deionized water and lyophilized. No activity was lost following these procedures. The kinetics of hydrolysis of RBBH by this lyophilized preparation were studied. The results showed that, using conditions described in Experimental procedures, the reaction is proportional to time for about 20 min and to protein concentration in the range of 0-20 μg/ml. The activity showed a pH maximum between 3 and 5. At pH 6.5-7.5 the enzyme exhibits about 10-15 % of the activity measured at pH 4.0. The temperature optimum is 36 °C. The activity is affected neither by the serine protease inhibitor PMSF, nor the metalloprotease inhibitor 1,10-phenanthroline, nor by trypsin inhibitor TLCK.
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Effect of CM on plasma membrane protein

Studies were undertaken to examine the effect of CM on the cells. For these experiments plasma membranes were isolated and purified (Rossomando & Cutler, 1975) and approximately 20 mg protein were incubated with catalytic amounts of CM. Following the incubation, the plasma membranes were removed from the reaction mixture by centrifugation and the polypeptides present in the reaction supernatant fractionated by electrophoresis on sodium dodecylsulphate-polyacrylamide gels. The products of each reaction were analysed on at least 2 gels and the reactions were performed using at least two different plasma membrane preparations.

Fig. 2 compares the peptide profiles obtained following incubation of CM with plasma membranes from either exponential growth phase cells (Fig. 2 A), stationary growth phase cells (Fig. 2B) or aggregation-competent cells (Fig. 2 c). The bands have been arbitrarily numbered for reference purposes. Also shown in Fig. 2 (broken line) are the control polypeptide profiles, that is, those polypeptides recovered following incubation of plasma membrane in the absence of CM. The polypeptides recovered in these control experiments probably represent peptides released by endogenous membrane-bound proteolytic activities.

Examination of Fig. 2A reveals that out of the approximately 26 polypeptide bands observed in the control, only bands 1 and 2 increase in the reaction supernatant after incubation with CM. In contrast, band 3 appears in the controls and does not change with addition of CM. Also, other peptides of lower molecular weight decrease in amount after treatment with CM.

Fig. 2B illustrates the peptide profiles obtained with plasma membranes from stationary phase cells. On examination of the high-molecular-weight region (the top third of the gel) only band 3 increases following incubation with conditioned media. In addition, band 2 is no longer observed. These results would suggest that any endogenous activity that might be present in vegetative membranes is probably absent from stationary phase membranes. The remaining two-thirds of the gel show a profile sufficiently similar to the vegetative pattern to suggest that while other changes have occurred in membrane proteins, none of these changes appear to be contingent upon incubation with CM.

Fig. 2C shows the polypeptide profile obtained following incubation of membranes from growth phase cells (A); stationary phase cells (B); and aggregation-competent cells (C); with (---) and without (---) conditioned medium.
prepared from aggregation-competent cells. Bands 1, 2 and 3 are barely detectable after incubation with CM.

In order to demonstrate that the changes in plasma membrane protein were due to the action of proteolytic activity present in the CM, control incubations were performed in which the protease substrate RBBH was added to the reaction mixture. The results of these experiments showed that upon incubation of membranes with CM plus RBBH the polypeptide profiles in Fig. 2A, B, C, were not obtained (data not shown).

![Graph showing recovery of cells after incubation on collagen-coated plates.](image)

**Fig. 3.** Recovery of cells after incubation on collagen-coated plates. Aggregation-competent cells and exponential growth phase cells were prepared as described in Methods. 0.4 ml of cells were placed on collagen-coated plates and allowed to incubate at room temperature for 30, 60 and 240 min. The supernatant was then removed and the plates washed with 0.4 ml of 0.1 M Tris-HCl (pH 7.5). The wash was removed and the numbers of aggregation-competent (△) and exponential (○) phase cells recovered in the supernatants were counted.

**Effect of CM on cell-substratum interactions**

The need to quantitate the effect of CM on cell-substratum interactions resulted in the selection of reconstituted rat-tail collagen as a substratum for the assay. Collagen-coated Petri dishes were prepared as described in Experimental procedures and overlaid with cells from different stages. At suitable intervals, the plates were washed with buffer and the number of cells recovered in the buffer wash was determined. These data are shown in Fig. 3 and they illustrate the following 2 points. First, the rate of detachment is stage specific. For example, during the first 0.5 h incubation about 90% of the vegetative cells remain attached to the substratum, whereas only about 60% of the aggregation-competent cells remain attached. Second, for cells from both stages, the numbers of cells that remain attached change with time. Thus, in the first 5 min
more than 90\% of both types of cells remain attached, but with time, both cell types become detached. These results show that cells from each stage can be characterized by their rate of release from the substratum, with the aggregation-competent cells having a faster rate of detachment than exponentially growing cells.

In order to determine the effect of conditioned medium on cell-substratum interactions, exponentially growing cells were spread on collagen-coated dishes together with CM and the rate of release of the cells from the collagen substratum determined. The results shown in Table 2 indicate that in the presence of CM there has been a decrease in the rate of release of the cells. Thus, CM appears to inhibit detachment.

**Table 2. Rate of release of growth phase cells from collagen substratum under various conditions**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cells released after 30 min, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
</tr>
<tr>
<td>CM† addition</td>
<td>10</td>
</tr>
<tr>
<td>RBBH‡ addition</td>
<td>50</td>
</tr>
<tr>
<td>CM + RBBH addition</td>
<td>30</td>
</tr>
<tr>
<td>Pre-incubation of collagen with CM for 30 min§</td>
<td>1–2</td>
</tr>
</tbody>
</table>

* 4 x 10⁶ vegetative stage cells were used in all experiments.
† CM was prepared as described in Methods; 0.4 ml was spread on collagen-coated plastic Petri dish before addition of cells.
‡ RBBH was dispersed together with cells over bottom of dish.
§ CM was spread over collagen substratum and remained for 30 min.

The effect of proteolytic activity on the attachment process was studied further by spreading the inhibiting substrate RBBH over the surface of the collagen before dispersing the vegetative cells. Again the number of cells released after 30 min was determined. The results, shown in Table 2, indicate that the rate of release of the cells has been increased, suggesting that the proteolytic activity functions extracellularly to slow the detachment process. Finally the effect of CM on detachment could be related to its proteolytic activity by incubating CM together with RBBH on the collagen-coated dish; the rate of release of the dispersed cells is greater than that observed with CM alone (Table 2). This result is consistent with the idea that the proteolytic activity in the CM is affecting the rate of release. To study the mechanism of action of the proteolytic activity in the CM further, collagen plates were preincubated with CM. After 30 min, the CM was removed, the plates were washed twice with buffer and cells dispersed over the surface. This pretreatment results in a significant decrease in the rate of release of the cells (Table 2). Thus, the detachment process has been slowed by the pretreatment of the collagen with the CM. Since the effect of CM occurs before the cells have been added at least 2 explanations are consistent with this result. Either the proteolytic activity present in the CM acts on the collagen to slow detachment or the CM contains components which ‘coat’ the surface of the collagen thereby slowing the detachment.
The effect of CM on multicellular-structure formation

To study these alternatives, untreated plastic Petri dishes were used, and an aliquot of the CM was spread over the bottom. After 60 min the CM was poured off and the bottom of the dish overlaid (again) with $4 \times 10^6$ cells. In the control dish, the bottom was covered with buffer overlaid with $4 \times 10^6$ cells. After a 4-h incubation at room
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Temperature, both the experimental and control dishes were examined microscopically. The bottom of the control dish appeared as shown in Fig. 4A. The cells are isolated and randomly dispersed with no evidence of multicellular structure. In contrast, when the dish pretreated with CM was examined, the cells appeared as shown in Fig. 4B. Large islands of cells can be seen reminiscent of the early stages of multicellular structure formation. The effect was not observed when bovine serum albumin was substituted for CM suggesting that this result is not due to coating of the plastic by a non-specific protein.

As described above, multicellular structures are formed with CM in the absence of collagen. This finding suggests that CM contains components which could coat the bottom of the plastic dish and promote the formation of multicellular structures. It was therefore of interest to determine if such components might be derived from intact cells as well. To study this possibility, $4 \times 10^6$ cells were first dispersed over the bottom of both the experimental and control dishes. Both the control and experimental dishes were examined microscopically at 30-min intervals. Cells on the experimental plate displayed an orientation suggestive of the multicellular structure seen with CM. The cells appeared to stick to one another in an end-to-end manner. In contrast, cells on the control plates were distributed at random in a pattern similar to the one shown in Fig. 4A. After an additional hour, the experimental plates showed large multicellular structures from which streams of cells arranged end-to-end emerged, while on the control plates the cells remained randomly dispersed. After 4 h, the experimental plates showed patterns illustrated in Fig. 4C. Clearly, multicellular structures have been formed. In contrast, cells on the control plates remained randomly dispersed over the surface. These results suggest that the cells leave behind a component on the plate which promotes structure formation. Finally, to obtain more

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Fig. 4. Effect of conditioned medium on aggregation in uncoated plastic dishes. A, approximately 0-4 ml of 0-1 M Tris-HCl (pH 7.5) was placed on a plastic Petri dish and the dish allowed to incubate for 1 h at room temperature. The buffer was poured off and approximately $4 \times 10^6$ cells (0-4 ml) were laid down on the dish and the dish incubated an additional hour. Cells were examined at 30-min intervals and photographs taken on a Zeiss photomicroscope II. Photomicrographs of unstained cells. $\times 250$.

B, approximately 0-4 ml of conditioned media (CM) was placed on plastic Petri dishes and the dish incubated for 1 h at room temperature. The CM was poured off and approximately $4 \times 10^6$ cells (0-4 ml) were laid down on the dish and the dish incubated an additional hour. Cells were examined and photographs taken as described in A above. $\times 250$.

C, approximately $4 \times 10^6$ cells (0-4 ml) were placed on plastic Petri dishes and the dish incubated for 1 h at room temperature. The supernatant was poured off and the plate washed until all the cells were removed. Cell concentration was determined with a haemocytometer. An additional $4 \times 10^6$ cells were laid down, the dish incubated, and cells examined and photographs taken as described above, $\times 100$.

D, approximately $4 \times 10^6$ cells (0-4 ml) were placed on plastic Petri dishes and incubated for 1 h at room temperature. The supernatant was poured off and the plate washed until all the cells were removed. Cell concentration was determined with a haemocytometer. The plates were examined and photographs taken as described above. $\times 250$. 
direct evidence for a cell residue on the plastic, uncoated plastic plates were layered with $4 \times 10^6$ cells and after 1 h, the buffer was poured off and the cells were recovered as described above. Immediately, the plates were examined under the microscope. A representative photograph is shown in Fig. 4D. While an occasional cell is observed, a cell residue is visible on the plastic, consistent with the proposal that cells leave behind a material which aids in the formation of multicellular structures.

DISCUSSION

When exponentially growing cells of the cellular slime mould Dictyostelium discoideum are incubated in a non-nutrient buffer for 6 h they become aggregation-competent (Lee, 1972).

The data presented in this study indicate that during the first 2–3 h in this buffer, a proteolytic activity is released by these cells. This activity is only marginally detectable in the medium of growing cell cultures but does appear extracellularly at significant levels when cells reach stationary phase.

Additional studies showed a similarity in the rate of release of the protease and 2 glycosidase activities. Since these particular glycosidases have been reported to be of lysosomal origin (Weiner & Ashworth, 1970), it is tempting to speculate that the proteolytic activity we have observed is of a similar origin. This conclusion derives support from the report that growing D. discoideum contain an acid protease (pH optimum 2 or less) of lysosomal origin (Ashworth & Quance, 1972), together with the finding that our proteolytic activity has an acid pH optimum.

To study the possible role of this proteolytic activity we have taken into consideration the fact that its release is correlated with the onset of development. However, since its release also coincides with the onset of starvation, a possible role in food acquisition should be considered. Such a function has been suggested for a proteolytic activity secreted by Tetrahymena (Dickie & Liener, 1962). However, in the case of D. discoideum, where food is usually ingested by phagocytosis and digested intracellularly, the secretion of proteases would not necessarily make available the nutrients required for growth.

Thus we considered the possibility that components at the cell surface might be the natural substrate. To approach this question, purified plasma membranes were incubated with CM. The results showed that polypeptides were released and that some variations existed in the susceptibility of the membranes from different stages of growth and development and that this release could be blocked by the substrate, RBBH. We conclude that the release is due to proteolytic activity present in the CM.

In order to show that the proteolytic activity present in the conditioned medium could modify the surface properties of D. discoideum, we developed an assay based on the differences in the rates of detachment of cells at different stages from a collagen substratum. Using this assay we were able to show that detachment from the coated plastic dish was accelerated by inhibition of the proteolytic activity. While the results of these studies strongly suggested a correlation between detachment and extracellular proteolytic activity, the possibility existed that the protease promoted detachment by
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acting on the collagen substratum and not by any effect on the cell. However, the results of our studies clearly show not only that CM can reduce the rate of detachment of the cells from uncoated plastic dishes but that a residue left behind on the plastic by cells can also promote the formation of multicellular structures. Cell products such as this residue have been seen in other systems and biochemical analysis has indicated that such 'footprints' contain numerous proteins including actin (Culp, 1976). It remains for further studies to determine whether the D. discoideum residue is of a similar nature.

Extracellular proteolytic activity has been implicated in development of chick embryo cells (Rutishauser et al. 1976), the transformation process (Rifken, Loeb, Moore & Reich, 1974) and in the turnover of 5’-nucleotidase in D. discoideum (Rossonando & Maldonado, 1976). From the results of the present study it would appear that the extracellular protease could be involved in mediating the transition from migration to multicellular structure formation. Although it might be premature to propose a mechanism underlying this mediation, these data suggest a sequence involving the secretion of the protease, its action on components of the plasma membrane, and the coating of the substratum with cell surface components. While additional details must await the outcome of subsequent experiments, this study suggests that the action of proteolytic activities on the cell surface might involve not only modification of the surface enhancing subsequent intercellular adhesive events but also the generation of components which, by coating the substratum, facilitate migration.

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