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Density sensing in *Dictyostelium discoideum*: Evidence for the sequestering of a density sensing factor in the plasma membrane and a role for this factor in cell aggregation

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DENSITY SENSING IN DICTYOSTELIUM DISCOIDEUM: EVIDENCE FOR THE SEQUESTERING OF A DENSITY SENSING FACTOR IN THE PLASMA MEMBRANE AND A ROLE FOR THIS FACTOR IN CELL AGGREGATION

by

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ABSTRACT

DENSITY SENSING IN *Dictyostelium discoideum*: EVIDENCE FOR THE SEQUESTERING OF A DENSITY SENSING FACTOR IN THE PLASMA MEMBRANE AND A ROLE FOR THIS FACTOR IN CELL AGGREGATION

by

Carl R. Taphouse

A density sensing factor has been isolated from *Dictyostelium discoideum* and is known as conditioned media factor (CMF). CMF has been shown to cause starved vegetative *Dictyostelium* cells to differentiate into one of two cell types, stalk or spore cells. One interesting aspect of CMF concerns its release: CMF mRNA is present in vegetative *Dictyostelium* cells, yet secretion of the CMF protein only occurs during early and late development. My research reveals evidence that CMF is sequestered in the plasma membrane of vegetative cells.

Further experiments show that CMF anti-sense transformants are motile but do not form cell aggregates. I suggest that CMF may have a role in chemotaxis which could involve interactions with components of the cytoskeleton. I have identified a few cytoskeletal components in vegetative and developing *Dictyostelium* cells which may provide useful information concerning the developmental process.
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I. Introduction

Cellular response to environmental signals is essential for proper tissue development. Communication signals not only provide information that allow a cell to determine its spatial orientation and structural morphology but do it in a temporally coordinated manner. Breakdowns in cellular communication can result in tissue death, developmental defects, as well as diseases such as cancer. Studies on cellular communication have been going on for decades, but only recently have such studies begun to provide insight at the molecular level. As discoveries are made, more effective treatments will become available to deal with developmental abnormalities.

Methods of Communication: Cell Migration

A cell uses communication signals to guide it to its appropriate position in a tissue. Migration is generally thought to be directed by chemical signals (chemotaxis and haptotaxis), ionic signals (galvanotaxis), or physical factors that can serve to guide (contact guidance) or restrain (contact inhibition) cell movement.

Chemotaxis has been studied in a number of organisms and is a consequence of cellular response to a concentration gradient of a chemical factor in solution. Early experiments (Bonner, 1947 and Shaffer, 1953) demonstrated the existence of a chemoattractant in the slime mold Dictyostelium discoideum that was later identified as cyclic adenosine 3',5'-monophosphate (cAMP) (Konijn et al., 1967; Bonner et al., 1969). Later
studies (Miller, 1978) have uncovered the existence of a species-specific sperm attractant in the eggs of cnidarian *Orthopyxis caliculata*. *Orthopyxis* eggs not only secrete a chemoattractant but regulate the timing of its release to assure that fertilization will occur at the appropriate time. Speract, a 10-amino acid peptide from *Strongylocentrotus purpuratus* (Hansbrough and Garbers, 1981) and Resact, a 14-amino acid peptide from *Arbacia punctulata* (Ward et al., 1985), have recently been isolated and identified as species-specific sperm attractants in sea urchin. Chemotaxis has also been observed in a strain of *Escherichia coli*, which aggregate together in response to oxygen deprivation by secreting an attractant that is sensed by the aspartate receptor (Budrene and Berg, 1991).

The differential adhesive specificity hypothesis suggests that a cell can also migrate in response to an adhesive molecule gradient in the extracellular matrix. Such guidance is known as haptotaxis and has been found to occur in the elongation of the pronephric duct in salamanders (Poole and Steinberg, 1982) and in axonal outgrowth in insects (Nardi, 1983). A recent study (Steinberg, 1987) suggests that the pronephric duct cells migrate in response to a gradient of alkaline phosphatase that is established across the mesodermal surface of the salamander embryo. Other studies (Akers et al., 1981; Gundersen, 1987; Erickson, 1988; Lehmann et al. 1990) have revealed similar roles for fibronectin gradients, laminin gradients, and lectin gradients in axonal growth and development. Trunk neural crest cells, for example, have been shown to migrate along the basal lamina in response to gradients of fibronectin and laminin. Very stringent conditions are required for a substance to serve as a
haptoattractant since variations in its adhensive strength could result in cell immobilization (if the attractant is too adhesive) or in failure of the substance to maintain a cell on its migration pathway (if the substrate is not adhesive enough).

Although not as well studied, galvanotaxis, or cell movement in response to an electric field, may also play an important role in cell migration (McCag, 1986). Nerve growth in *Xenopus laevis* has been demonstrated to be effected by electric fields that measure as little as 7 mV/mm (Hinkle et al. 1981; Jaffe and Poo, 1979; Gruler and Nuccitelli, 1991). Larger electric currents have been detected in early chick embryos (Jaffe and Stern, 1979) and in the ovary of the *Cecropia* moth (Woodruff and Telfer, 1974), where galvanotaxis has been suggested as the mechanism for selective transport of materials from the follicle cells into the oocyte. While it is not known how these potential differences direct cell migration, one hypothesis suggests that the establishment of an electric field may cause an influx of Ca$^{+2}$ into a localized region, triggering cytoskeletal assembly and subsequent cellular motility (Cooper and Schliwa, 1985; Soong *et al.* 1990; Ueda *et al.* 1990). This phenomenon may account for the effectiveness of electrical stimulation in wound healing (Reich *et al.* 1991).

In addition to being directed by chemical and ionic factors, a cell is also guided and restrained by physical elements it encounters during migration. Contact guidance was elegantly demonstrated by Stopak and Harris (1982) using embryonic chicken fibroblasts. Their studies showed that fibroblasts cultured on collagen were capable of creating stress folds
(stress fibers) in the collagen substrate and that the stress folds could be used to direct cell movement. Utilizing a collagen-coated plate conditioned with fibroblasts, they were able to get randomly scattered muscle cells to form functional units by aligning with the stress folds. Experiments (Clark et al. 1990) using titanium-coated micromachined surfaces suggest that groove depth is the dominant factor effecting cell alignment.

Cells are also restrained in their movement by steric hinderance, as evidenced by neural crest cell migration (Newgreen, 1989), and by a process known as contact inhibition. Contact inhibition results in the migration of motile cells away from areas of high cell concentration. During migration, a mesenchymal cell moves by extending a process known as a lamellipodium. When the lamellipodium makes contact with a neighboring cell it becomes paralyzed and disappears. A new lamellipodium then forms, directing the cell away from the point of contact. Contact inhibition is evident in the process of wound healing. Radice (1980) found that when epidermal cells are removed from *Xenopus* tadpole tails, the resulting wound area is rapidly covered by underlining basal cells. The basal cells, normally sessile, have been found to form lamellipodia within 5-10 seconds and tranverse the basement membrane until they make contact with each other and stop moving again. Loss of contact inhibition is seen in cancer cell lines, partly accounting for their invasive behavior.
Methods of Communication: Cell Differentiation

In addition to the many migratory signals that effect cell movement, a cell also responds to factors that trigger morphological change. These factors govern the synthesis and secretion of proteins, control cell growth and division, and regulate fluid levels within the cell. Like chemotactic substances, many of these morphological factors (morphogens) are believed to exert their effects by establishing gradients within a given cell population. Others act through proximate interactions between adjacent cells.

Morphogen gradients have been identified and studied extensively in the fruit fly *Drosophila melanogaster*. During early oogenesis, products of the maternal genome are placed in the oocyte that specify germ line production and establish the dorsal-ventral and anterior-posterior body axes. These maternal-effect gene products form gradients by interacting with components of the egg cytoplasm and exert their effects by activating and repressing zygotic gene expression in a concentration-dependent manner. Mutant rescue experiments have identified a number of these gene products, including the *Toll* gene product, which is responsible for establishing the dorsal-ventral axis, and the *bicoid* gene product, which is responsible for specifying anterior-posterior polarity (Anderson *et al.* 1985; Struhl *et al.* 1989; MacDonald and Struhl, 1988; Driever and Nusslein-Volhard, 1988).

Similar morphogenic gradients have also been discovered in *Dictyostelium discoideum, Hydra pirardi*, and in the developing chick limb bud. In *Dictyostelium*, differentiation-inducing factors (DIFs) have been
implicated in the induction of prestalk cell formation and in the repression of prespore formation (Kwong et al. 1990; Kay and Jermyn, 1983; Morris et al. 1987). In hydra, activator and inhibitor gradients have been postulated and identified that are responsible for establishing body polarity (Browne, 1909; Newman, 1974; Webster and Wolpert, 1966; MacWilliams, 1983a,b; MacWilliams and Kafatos, 1974; Hicklin and Wolpert, 1973; Grimmelikhuijzen and Schaller, 1977; Schmidt and Schaller, 1976; Schaller et al., 1979; Berking et al., 1979). In chick limb bud development, retinoic acid, the active form of vitamin A, appears to act as a morphogen in digit pattern development by forming a concentration gradient across the limb bud (Tickle, 1981; Thaller and Eichele, 1987; Tickle et al., 1985; Maden and Summerbell, 1986; Eichele et al. 1985; Maden et al. 1988).

Gradient models work well for explaining morphallaxis development, development involving rearrangement and respecification of existing cell populations, but when growth requires proliferation of new cells, a process known as epimorphic development, a different model is required. French (1976) and Bryant (1981) have proposed the polar coordinate model to help explain this type of development. The polar coordinate model suggests that a cell obtains positional information by examining proximate relationships with adjacent cells and uses this information to specify its type. The model is well suited for explaining limb regeneration in the salamander.

When a salamander limb is amputated epidermal cells migrate to the wound and cover it with a single layer of cells known as the apical
ectodermal cap. Cells lying under the apical ectodermal cap undergo a process of dedifferentiation, resulting in the formation of a regeneration blastema. Cells contained in the regeneration blastema are returned to an embryonic state of development, and each is capable of differentiating into any of the cell types needed for limb regeneration. According to the polar coordinate model, cells in the blastema receive positional information from adjacent cells located proximal to the site of the wound, suggesting that proper regeneration of the limb could be disturbed by placing normally nonadjacent tissues next to each other at the wound site. Blastema rotation experiments (Bryant and Iten, 1976), where freshly-excised blastema are rotated 180 degrees relative to each of their stumps, results in limbs containing supernumerary structures, providing support for the polar coordinate model.

**Communication Using Density Sensing**

To form higher ordered biological structures, cells must be able to interpret and respond to spatial cues from their environment, including cues that provide information about their density. One way cells can sense their density is by secreting autocrine factors which they can simultaneously sense and respond to in a dose dependent manner. Such factors have been identified in *Myxococcus xanthus* (Kim and Kaiser, 1990a,b), *Vibrio fischeri* (Eberhard et al., 1981), *Bacillus subtilis* (Grossman and Losick, 1988), and *Dictyostelium discoideum* (Mehdy et al. 1983). Understanding how density sensing factors work in these simple
organisms could provide clues to the mechanisms of tissue size
determination.

Because of its simple life cycle (Figure 1), the slime mold,
*Dictyostelium discoideum*, serves as an ideal model for studying eukaryotic
growth and development. Development commences approximately 6 hours
after starvation when individual amoebae, responding to pulses of cAMP (a
chemoattractant), stream together forming cell aggregates of $\sim 10^5$ cells.
Each aggregate forms a slug or pseudoplasmodium, which migrates to a
favorable environment and begins differentiating into a fruiting body
containing stalk and spore cells.

The complete developmental cycle takes from 24 to 26 hours, but
cell differentiation can be detected as early as 10 hours after starvation in
prestalk cells and 15 hours after starvation in prespore cells by monitoring
for the presence of pst-cathepsin (a prestalk protein) and SP70 (a prespore
protein), respectively. An early study (Bonner, 1957) suggested that the
decision to become a stalk cell or a spore cell was dependent upon the
position of the cell in the slug with anterior cells becoming stalk cells and
posterior cells becoming spore cells. This situation was found to be true
even if the anterior of the slug was removed; cells in the new anterior,
previously destined to become spore cells, would change their fate and
become stalk cells. Further experimental evidence, however, indicates that
cell fate is initially determined by cell cycle phase at the time of starvation
Figure 1. The developmental life cycle of Dictyostelium discoideum.

Following starvation, unicellular vegetative amoebae aggregate together using cAMP-mediated chemotaxis. The resulting pseudoplasmodium undergoes differentiation to form a mature fruiting body composed of stalk and spore cells. When conditions are favorable, spores are dispersed and germinate into unicellular vegetative amoebae, which grow and divide until starvation conditions prompt another developmental cycle.
(Weijer et al., 1984; McDonald, 1986; Gomer and Firtel, 1987); later changes are enacted to preserve the slug cell-type ratio by modulating factors such as adenosine, ammonia, oxygen, and chlorinated hydrocarbons (Gross et al. 1983; Schapp and Wang, 1986; Brookman et al. 1987; Williams et al. 1987; Sternfeld, 1988; Kwong and Weeks, 1989; Xie et al. 1991). The cell cycle studies may suggest a need for cell sorting during aggregation. Malcolm Steinberg (1964) proposed a thermodynamic model of cell sorting that might best explain this phenomenon. In his model, a cell aggregate strives to attain a state of smallest interfacial free energy. In other words, if homotypic adhesions (adhesions between identical cell types) are stronger than heterotypic adhesions (adhesions between nonidentical cell types) sorting will occur until both cell types are separated from each other. Conversely, if homotypic adhesions are weaker than heterotypic adhesions, no sorting will occur. Since prestalk and prespore cells are known to exist as separate populations within the slug, cell cycle fate determination suggests that cell surface adhesion molecules may be present on prestalk and prespore that distinguish the 2 cell types from each other and favor homotypic adhesions over heterotypic adhesions.

Knowing when to express developmental genes is critical for proper fruiting body formation. In Dictyostelium discoideum, prestalk and prespore genes do not get expressed until after aggregation is completed. Studies focusing on the role of cAMP in aggregation and cell differentiation have led to the discovery of two intracellular signal transduction pathways that are activated by the binding of cAMP to cell surface receptors. One pathway serves to amplify the cAMP signal by
activation of adenylate cyclase (Devreotes, 1982; Gerisch, 1987; Jansssens and Van Haastert, 1987; Kessin, 1988). The other pathway activates phospholipase C, which is believed to be involved in initiating chemotaxis and, after aggregation, in inducing prespore and prestalk gene expression (Jansssens and Van Haastert, 1987; Mann and Firtel, 1987; Mann et al. 1988).

Recent experiments (Mehdy et al. 1983; Mehdy and Firtel, 1985) have provided evidence for the existence of an additional group of extracellular molecules that are required for the induction of developmentally-regulated genes. These molecules, collectively known as conditioned medium factors (CMFs), appear to play a role in sensing cell density. Under normal conditions, cells starved at low density (less than $5 \times 10^3$ cells cm$^{-2}$) are found to be incapable of prestalk or prespore gene expression, even if supplemented with high levels of cAMP. Induction will proceed, however, if these low density cells are treated with buffer previously conditioned by starving high density cells (conditioned medium). This conditioned buffer has, subsequently, been shown to contain two classes of CMF molecules, both of which, along with cAMP, are capable of inducing prestalk and prespore gene expression. One class represents the $80 \times 10^3$ $M_r$ high molecular weight CMF molecule (CMF-H), the other class represents a set of smaller (less than $10 \times 10^3$ $M_r$), more efficacious CMF molecules (CMF-L). Both classes of the molecule are protease-sensitive and appear to be N-linked glycosylated and O-linked glycosylated (Yuen et al. 1991). In fact, CMF-H has been found to
breakdown to CMF-Ls, as well as, to an active $65 \times 10^3$ M, form, suggesting that both classes originate from a common precursor.

A common mRNA has been discerned in northern blots from vegetative cells and during early development in *Dictyostelium discoideum* (Jain et al. in press). Since CMF is not secreted in vegetative cells (Mehdy and Firtel, 1985), this observation suggests that CMF might be sequestered in vegetative cells prior to release during early development. My research efforts have revealed evidence that supports this hypothesis.

Additional evidence also proposes a role for CMF in chemotaxis since CMF anti-sense transformants, while still motile, do not aggregate under starvation conditions. Coordinated movement in response to chemotactic signals, undoubtedly, involves components of the cytoskeleton, a few of which I have isolated and partially characterized.

II. Materials and Methods:

**Membrane Preparation and Conditioned Medium Assay**

CMF activity was measured using a modified method derived from an immunofluorescence assay (Gomer et al. 1986a,b) that measures SP 70 expression in developing low density *Dictyostelium* cells. CMF activity is defined as the dilution of material at which SP 70 expression is 50% maximum; 100 units ml$^{-1}$ of activity would represent 50% SP 70 expression at a 100-fold dilution of material.
Plasma membranes were isolated as outlined by Goodloe-Holland and Luna (1987) using a method developed by Das and Henderson (1983). Briefly, 1 x 10^9 vegetative cells were harvested and washed in lysis buffer (0.5 mM CaCl_2, 0.5 mM MgCl_2, 5 mM glycine, pH 8.5 at room temperature and pH 9.0 at 0°C). Washed cells were quickly warmed to room temperature, lysed by passage through a 5 μm pore size filter, and centrifuged at 5,900 g for 20 minutes at 2°C. The membrane-containing pellet was layered on a 0.75 to 1.5 M sucrose gradient in 50 mM glycine, pH 8.5 and centrifuged at 4°C for 18 hours at 145,000 g. Separated membrane fractions, sucrose gradient pellets, and lysate supernatant were assayed for CMF activity at dilutions of 1:10, 1:100, 1:10^3, and 1:10^4 by subjecting starved low density cells to the diluted material and monitoring for the presence of SP 70 protein using immunofluorescent microscopy. A hand refractometer (Fisher, Pittsburgh, PA) was used to measure the sucrose concentrations of a set of standards prepared in the glycine buffer. The standards were used to determine the sucrose concentration of the only band showing quantifiable levels of CMF activity.

Components of the CMF-containing fraction were separated on a SDS-10% polyacrylamide gel next to a lane containing relative molecular mass standards. Following electrophoresis, the lane containing the standards was cut from the gel and stained with Coomassie blue and used to construct a plot of log (relative molecular mass) vs. migration distance. The lane containing the membrane sample was soaked in distilled water for 5 minutes to remove SDS and cut into 2.5 mm sections. Each section was eluted overnight in 1 ml of PBM at 12°C and assayed for CMF activity.
The relative molecular mass of the section containing CMF activity was determined by extrapolating from the standards plot and was found to be between 80 kilodaltons and 90 kilodaltons.

**Dictyostelium** Cytoskeleton Preparation, Isolation, and Identification

For the cytoskeletal enrichment protocol, nine liters of **Dictyostelium** cells, grown at 22°C in HL-5 media, were harvested from shaking culture at a density of 2.5 x 10⁶ cells ml⁻¹. Harvested cells were centrifuged at 500 g for 15 minutes and suspended by manual stirring for 5 minutes in 250 ml of ice cold PDF buffer (2 mM KCl, 0.9 mM K₂HPO₄, 1.3 mM NaH₂PO₄, 0.25 mM MgCl₂, 5 mM EGTA, titrated to pH 6.5). For the developing cell cytoskeleton preparation, the harvested cells were washed in 500 ml of PBM buffer (22°C), centrifuged at 500 g for 15 minutes, suspended in 150 ml of PBM buffer (22°C), plated out on 1.4% (in water) agar plates to a density of 1 x 10⁸ cell ml⁻¹ and allowed to develop for 15 to 20 hours before being suspended in the ice cold PDF buffer, as above. Washed cells were pelleted by centrifugation at 500 g for 10 minutes. All subsequent operations were performed at 4°C, using buffers titrated to pH 7.6 (pH 7.2 for the urea extraction buffer), containing 0.1% 2-mercaptoethanol and protease inhibitors (0.25 mM o-phenanthroline, 0.2 mM TAME, 0.1 mM benzamidine, 1 μM leupeptin, 0.2 mM PMSF, 1 μM pepstatin). After each extraction (extractions carried out by stirring on a magnetic stir plate), residues were recovered using centrifugation at 13000 g and suspended using gentle dounce homogenization. Samples of supernatants and pellets
were collected after each extraction step for further analysis. The cells were lysed by extracting twice in 50 ml of LISB buffer (5 mM Tris-HCl, 4 mM MgCl₂, 5 mM EGTA, 2.0% NP-40); once for 15 minutes, followed by 20 minutes of centrifugation, and once for 10 minutes, followed by 1 hour of centrifugation. The resulting pellet was extracted for one hour in 50 ml of KCl buffer (1.5 M KCl, 5 mM Tris-HCl, 4 mM MgCl₂, 5 mM EGTA, 0.5% NP-40) and then centrifuged for one hour before being extracted in an additional 50 ml of KCl buffer overnight. The following day, the residue was collected by centrifugation for 90 minutes and extracted in 25 ml of KI buffer (0.6 M KI, 5 mM Tris-HCl, 4 mM MgCl₂, 5 mM EGTA, 0.5% NP-40) for 20 minutes. After one hour of centrifugation, the pellet was extracted a second time in 25 ml of KI buffer for 2.5 hours and centrifuged for an additional one hour. The resulting residue was washed in detergent-free LISB buffer for 2.5 hours and subjected to another hour of centrifugation. The final pellet was suspended in 1.5 ml of urea extraction buffer (6 M urea, 10 mM imidizole, 5 mM EGTA) and rotated in an Eppendorf tube overnight at 4°C and centrifuged for 1 hour at 15,000 x g.

Techniques for polyacrylamide gel electrophoresis were based on procedures of Laemmli (1970). Samples of supernatants and pellets taken during cytoskeleton preparation were denatured using SDS-sample buffer (50 ml of 5X stock: 250 µl 200 mM EDTA, 1.925 g DTT, 12.5 ml 20% SDS, 10.0 ml 1 M Tris-HCl, pH 6.8, 12.5 ml glycerol, pinch of bromphenol blue, water to 50 ml). Molecular weight markers purchased from Sigma Chemical Company (Sigma SDS-6H molecular weight
markers) were electrophoresed in conjunction with the sample preparations on 10% SDS gels. Western blotting of gels to PVDF membrane was performed using a modified protocol based on methods described by Yuen et al. (1989). Bands of interest were cut from the membrane and digested with trypsin overnight at 37°C. Peptide separation was done on a 4.6 x 250 mm C18 reverse phase HPLC column. Fractions were collected by hand and concentrated using lyophilization. Peptides of interest were sequenced using Edman degradation (Konigsberg, 1972).

**Dictyostelium Motility Studies**

Fifteen milliliters of *Dictyostelium* cells, growing at a density of less than $1 \times 10^5$ cells ml$^{-1}$, were harvested from shaking culture by centrifuging at 500 g for 5 minutes and resuspending in 1 ml of PBM buffer (20 mM KH$_2$PO$_4$, 1 mM MgCl$_2$, 0.01 mM CaCl$_2$, pH 6.5 at 22°C). Resuspended cells were added to a 60 x 15 mm plastic petri dish containing 5.68 ml of PBM to give a final cell density of $4 \times 10^3$ cells ml$^{-1}$. Time lapsed videos (taken at 1 frame every 4 seconds) of amoebae movement were made by observing cells under a phase contrast microscope. Migration distances were determined by measuring net distance traveled from 0 to 4 hours and from 4 to 10 hours.
III. Results

Sequestration of Conditioned Medium Factor in the Plasma Membrane of Vegetative Dictyostelium Cells

Early studies showed that conditioned medium factor (CMF), a density sensing molecule, is secreted by Dictyostelium cells during the first 5 hours of development and again throughout late development but not during vegetative growth (Mehdy and Firtel, 1985). Northern blots of cellular messenger RNA, however, reveal the presence of CMF mRNA transcripts in vegetative cells (Jain et al., in press). The general instability of messenger RNA (Nevins, 1983; Darnell, 1982) suggests that CMF might be stored in the cell for later secretion. We demonstrate that CMF is sequestered in the plasma membrane of vegetative cells.

Plasma membranes were prepared by the method of Das and Henderson (1983) (Figure 2) as outlined by Goodloe-Holland and Luna (1987). Separation of plasma membranes from other subcellular membranes was carried out using a sucrose gradient that resulted in one band designated by Das and Henderson as the "combined 1 and 2 bands" showing significant levels of CMF activity (Table 1). This band (E) was present in the sucrose gradient at a concentration of 1.28 M sucrose and showed a protein profile, when run on an SDS-polyacrylamide gel, essentially the same as that illustrated in Fig. 5 of Goodloe-Holland and Luna (1987) (Figure 3).
Figure 2. Flow diagram for plasma membrane preparation using the method of Das and Henderson (1983). DH (Das and Henderson profile), T (Results obtained by Taphouse).
Washed cells

Lysed through Nucleopore filter in Calcium, Magnesium, pH 8.5

Pellet  Supernatant

Wash

Sucrose Gradient, pH 8.5
(145,000 g, 18 h, 2°C)

Load

0.75 M to 1.5 M Gradient

DH

T

DH3

DH2

DH1

Collect

Wash
Table 1. Data from conditioned medium factor assay.

Starved low density vegetative *Dictyostelium* cells were treated with cytosol and membrane fractions collected from the Das and Henderson plasma membrane preparation and assayed for the expression of SP 70 protein using immunofluorescence at dilutions of 1:100, 1:1000, 1:10,000. The 1:1000 dilutions and the 1:10,000 dilutions did not show activity. Of the fractions assayed at 1:100, band E (corresponding to the Das and Henderson "combined 1 and 2 band) showed the maximum levels of CMF activity. Postive control, crude conditioned medium (medium taken from high density cells 20 hours after starvation in PBM); negative control, PBM; sucrose control, 1 M sucrose in 50 mM glycine, pH 8.5.
Number of Cells Expressing SP 70 Protein

Assayed at 1:100, 1:1000, 1:10000 dilutions

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<th>1:100</th>
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<td>Sucrose Control</td>
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Figure 3. The E membrane fraction collected from the *Dictyostelium* plasma membrane preparation showed maximum levels of CMF activity. This band (E) shows a protein profile, when run on an SDS-polyacrylamide gel, essentially the same as that illustrated in Fig. 5 of Goodloe-Holland and Luna (1987) for the membrane fraction Das and Henderson designate as the "combined 1 and 2 bands". S, molecular mass markers; A-F, membrane fractions collected from sucrose gradient.
A second sample of the sucrose band containing conditioned medium factor activity was subjected to electrophoresis on an SDS-polyacrylamide gel and divided into slices, which were tested for CMF activity as described (Gomer et al. 1991). One slice of the gel showed maximum levels of CMF activity (Table 2 and Figure 4); this slice cooresponded to a molecular mass of between 84 and 91 x 10^3 M_r, roughly equivalent to the molecular mass of CMF-H (Gomer et al. 1991), suggesting that vegetative cells might sequester CMF in the plasma membrane. However, given the difficulties inherent in membrane separation, it may be possible that the activity is associated with an intracellular membrane. To rule out the possibility that another molecule was mimicking CMF and causing the activity, the experiment was repeated using a CMF anti-sense mutant. In the anti-sense mutant study no detectable levels of CMF activity were observed (Table 3), demonstrating that CMF was responsible for the noted activity.

**Preliminary Characterization of Dictyostelium discoideum**

**Cytoskeletal-Associated Proteins**

A standard protocol was used to isolate cytoskeletal components from vegetative and developing Dictyostelium cells. Extracts and residues were collected at all stages of the preparation and subjected to electrophoresis on SDS-polyacrylamide gels (Figures 5: Vegetative Cells and 6: Developing Cells). Initial extraction with physiological buffer
Table 2. Data from conditioned medium factor assay.

A sample of the membrane fraction containing CMF activity was subjected to SDS-polyacrylamide electrophoresis. After electrophoresis, the gel was cut into 0.25 cm sections and the individual sections were assayed for CMF activity at dilutions of 1:100 and 1:1000. The 1:100 dilution did not show quantifiable levels of CMF activity, suggesting that CMF is inhibitory at this higher concentration. The section showing maximum levels of CMF activity at a dilution of 1:1000 was section #16. Sections measured from bottom of stacking gel. Positive control, crude conditioned medium (medium taken from high density cells 20 hours after starvation in PBM); negative control, PBM; sucrose control.
Number of Cells Expressing SP 70 Protein

Assayed at 1:1000 dilution

<table>
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<th># of Cells</th>
<th>Section #</th>
<th># of Cells</th>
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Figure 4. Crude plasma membranes from vegetative Dictyostelium discoiduem were subjected to electrophoresis on an SDS-polyacrylamide gel. Following electrophoresis, the gel was rinsed briefly and divided into 0.25 cm slices. Elutant from the slices was assayed for conditioned medium factor activity using a series of dilutions. Maximum activity was observed in sliced #16, which migrated 4 centimeters into the running gel. Relative molecular mass standards, run in an adjacent lane, were used to determine the relative molecular mass of the slice showing activity.
Table 3. Data from anti-sense mutant CMF assay.

Starved low density vegetative *Dictyostelium* cells were treated with cytosol and membrane fractions collected from the Das and Henderson plasma membrane preparation of CMF anti-sense mutants and assayed for the expression of SP 70 protein using immunofluorescence at dilutions of 1:10, 1:100, 1:1,000. No significant levels of CMF activity were noted in any of the fractions. Positive control, crude conditioned medium (medium taken from high density cells 20 hours after starvation in PBM); negative control, PBM; sucrose control, 1 M sucrose in 50 mM glycine, pH 8.5.
Number of Cells Expressing SP 70 Protein

Assayed at 1:10, 1:100, 1000 dilutions

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<td>Sucrose Control</td>
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Figure 5. Vegetative *Dictyostelium discoideum* cells were subjected to a step-wise series of salt extractions to enrich for insoluble cytoskeletal components. Fractions were collected at each stage of the preparation and separated on an SDS-polyacrylamide gel. Lane 1 (STDS), relative molecular mass markers; the relative molecular masses x 1000 are indicated. Lanes R1-E2, residues (R) and extracts (E) collected from extraction in LISB (physiological) buffer. Lanes R3-E4, residues (R) and extracts (E) collected from extraction in 1.5 M KCl buffer. Lanes R5-E6, residues (R) and extracts (E) collected from extraction in 0.6 M KI buffer. Lanes R7-E7, residues (R) and extracts (E) from extraction in LISB buffer (containing no detergent). Lanes R8-E8, urea residue (R8) and urea extract (E8). Arrow points to band with sequence identity to elongation factor 1-A (EF-1A).
Vegetative Cell Cytoskeleton Preparation

STDS R1 E1 R2 E2 R3 E3 R4 E4 R5 E5 R6 E6 R7 E7 R8 E8

205 116 97.4 66 45 29

EF-1A
Figure 6. Developmental *Dictyostelium discoideum* cells were subjected to a step-wise series of salt extractions to enrich for insoluble cytoskeletal components. Fractions were collected at each stage of the preparation and separated on an SDS-polyacrylamide gel. Lane 1 (STDS), relative molecular mass markers; the relative molecular masses x 1000 are indicated. Lanes R1-E2, residues (R) and extracts (E) collected from extraction in LISB (physiological) buffer. Lanes R3-E4, residues (R) and extracts (E) collected from extraction in 1.5 M KCl buffer. Lanes R5-E6, residues (R) and extracts (E) collected from extraction in 0.6 M KI buffer. Lanes R7-E7, residues (R) and extracts (E) from extraction in LISB buffer (containing no detergent). Lanes R8-E8, urea residue (R8) and urea extract (E8). Arrow points to band with sequence identity to discoidin I, A chain (D).
Developing Cell Cytoskeleton Preparation
removed large quantities of low molecular weight proteins (less than 100 kdaltons), which are highly soluble under physiological conditions, as well as some actin (42 kdaltons) and actin-binding proteins (lanes E1 and E2). KCl buffer extraction solubilizes large quantities of myosin heavy chain (200 kdaltons) and significant amounts of actin and actin-associated proteins (lanes E3 and E4). Most of the remaining actin and myosin was removed during extraction with KI buffer (lanes E5 and E6) leaving a highly enriched cytoskeleton residue, which was washed once in physiological buffer (lane E7) and extracted with 6 M urea to release most of the cytoskeletal proteins (lane E8), which are highly soluble in urea. The urea extracts and residues contained several proteins with molecular masses of approximately 50 kilodaltons, suggesting that they might be intermediate filament proteins. Since no intermediate filaments have been identified in *Dictyostelium*, I elected to sequence these proteins and check for similarity with known intermediate filaments found in higher eukaryotes. Peptides obtained from trypsin digestion of these proteins were separated using reverse-phase HPLC (Figures 7 and 8) and subjected to Edman degradation (see Appendix B for list of all sequences). Although none of the proteins sequenced showed similarity to intermediate filaments, two had matches with previously sequenced proteins. In the vegetative cell preparation one of the components of the urea residue showed similarity to elongation factor 1-alpha (Figure 9), a component of the protein synthetic machinery. This result corroborates earlier findings (Yang *et al.* 1990) and suggests that the cytoskeleton may be involved in protein synthesis,
Figure 7. The urea residue from the vegetative *Dictyostelium* cytoskeleton preparation was subjected to electrophoresis on an SDS-polyacrylamide gel and electroblotted to PVDF membrane. A protein band migrating with a relative molecular mass of 50 kD was cut from the membrane and digested with trypsin yielding a mixture of peptides that were separated using reverse-phase HPLC. The arrow points to a peak made by a peptide fragment that was found to be identical in sequence to a portion of the elongation factor 1-A protein.
Figure 8. The urea extract from the developmental *Dictyostelium* cytoskeleton preparation was subjected to electrophoresis on an SDS-polyacrylamide gel and electroblotted to PVDF membrane. A protein band migrating with a relative molecular mass of 36 kD was cut from the membrane and digested with trypsin yielding a mixture of peptides that were separated using reverse-phase HPLC. The arrows point to 2 peaks made by peptide fragments that were found to be identical in sequence to portions of discoidin I, A chain protein.
Figure 9. Peptide fragments derived from a 50 kD protein obtained from the vegetative *Dictyostelium discoideum* cytoskeleton preparation were separated using reverse-phase HPLC and subjected to Edman degradation. One of the separated peptides had a 16 amino acid sequence identity to elongation factor 1-A.
Elongation Factor 1-A Sequence Identity

EPKRPHDKPLRIPLQDVYKIGGIGTVPVG

VETGIKPGMVVTFAP

RVETGIKPGMVVTFAPAGLSTEVKSVEM

HHEQLPEARP GandNVGFNVKNSVKEIKR

GMVAGDSKNDPPQETEKFVAQVIVLNHP

GQIHAG
possibly serving as a scaffolding for localizing translational events (see also Hinkle et al. 1981, Jeffery, 1982, Howe and Hershey, 1984, Moon et al. 1983, Bagchi et al. 1987). In the developing cell preparation one of the components of the urea extract, which was not detected in the vegetative preparation, showed sequence identity to a region within a molecule belonging to a class of adhesion factors found in Dictyostelium known as discoidins (Figure 10). Suprisingly, my isolate migrates at approximately 36 kdaltons on an SDS-polyacrylamide gel while the previously identified Dictyostelium discoidins have approximate molecular masses of 26 kdaltons (discoidin I) and 24 kdaltons (discoidin II). This difference, however, may be due to differences in glycosylation.

CMF Anti-Sense Mutant Motility

CMF anti-sense mutants do not form cell aggregates under starvation conditions (Gomer, unpublished). At first, it seemed likely that this inability was the result of cellular paralysis. Preliminary findings, however, show that cells from both mutant and wild-type populations have roughly equivalent speeds (Figure 11 and 12) and appear to use similar propulsion mechanisms for cell movement. Speeds were determined by measuring net migration distances for wild-type and CMF anti-sense mutant cells in low density cell populations from 0 to 4 hours and from 4 to 10 hours at low magnification. Propulsion mechanics were compared by observing cell shape changes at high magnification. The low magnification
Figure 10. Peptide fragments derived from a 36 kD protein obtained from the developmental Dictyostelium discoideum cytoskeleton preparation were separated using reverse phase HPLC and subjected to Edman degradation. One of the separated peptides had a 14 amino acid sequence identity to discoidin I, A chain.
Discoidin I, A Chain Sequence Identity

TQPVQSSVTQVGADIYTGDNICALNTGSGK

EVVVPVKFQFEFAT
***************
REVVVPVKFQFEFATLPKVALNFDQIDCT

DATNQTRIGVQPRNITTKGFDCVFYTWNE

NKVYSLRADYIATALE
Figure 11. Vegetative *Dictyostelium* cells, growing at a density of less than 1 x 10^5 cells ml^{-1}, were harvested and resuspended in PBM buffer to a final cell density of 4 x 10^3 cells ml^{-1}. Time lapsed videos were made of amoebae movement by observing the cells under a phase contrast microscope. The migration distance for each cell was determined by measuring the distance between its position at 0 hours and its position at 4 hours.
Figure 12. Vegetative *Dictyostelium* cells, growing at a density of less than 1 \( \times 10^5 \) cells ml\(^{-1} \), were harvested and resuspended in PBM buffer to a final cell density of 4 \( \times 10^3 \) cells ml\(^{-1} \). Time lapsed videos were made of amoebae movement by observing the cells under a phase contrast microscope. The migration distance for each cell was determined by measuring the distance between its position at 4 hours and its position at 10 hours.
studies from 0 to 4 hours show large variances in speed in both mutant and wild-type populations suggesting random cell movement. In the 4 to 10 hour studies, however, mutant cells appear less motile than the wild-type cells. This difference may be due to directed movement of the wild-type cells in response to chemotactic signaling and suggests a role for CMF in the chemotactic process.

IV. Discussion

I have found evidence for the sequestering of conditioned medium factor (CMF) in vegetative Dictyostelium cells. Membrane purification procedures of Das and Henderson (1983) suggest that sequestered CMF is localized to the plasma membrane. However, because it is difficult to separate plasma membrane from other subcellular membrane components using this procedure, the possibility exists that CMF may be stored in subcellular vesicles. Such sequestration may explain why a different membrane purification scheme (Goodloe-Holland and Luna, 1987), which utilized Con-A capping of extracellular surfaces to separate out plasma membrane, failed to detect CMF activity in vegetative cells.

Although sequestering of CMF in vegetative cells does not rule out the possibility of de novo synthesis during development, calculations (Yuen et al. 1991) suggest that there are sufficient levels of CMF protein sequestered in vegetative cells to account for all observed secretion during Dictyostelium development. While it is not known why CMF is
sequestered in vegetative cells, energy considerations make it more practical to produce CMF during times when nutrients are abundant. Since development proceeds under starvation conditions, it makes more sense to synthesize CMF before it is actually needed.

Research efforts have generated a growing body of evidence that suggests a role for the cytoskeleton in localization and segregation of gene products during development. Such associations have been proposed in the segregation of Vg1 mRNA in *Xenopus laevis* (Yisraeli, 1990), the subcellular localization of histone mRNA in HeLa S3 cells (Zambetti, 1985), and in the positioning of maternal-effect gene products in the oocyte of developing *Drosophila melanogaster* (Bearer, 1991), to name just a few. The isolation of a cytoskeletal-associated protein from vegetative cells with sequence identity to elongation factor 1-A suggests a means for spatial regulation of protein synthesis during *Dictyostelium* development since elongation factor 1-A incorporation into the cytoskeleton has been shown to be regulated during chemotaxis (Dharmawardhane *et al.* 1989).

Conditioned medium factor (CMF) has been shown to be essential for inducing prestalk and prespore gene expression in *Dictyostelium*. Recent studies of CMF anti-sense mutants, however, indicate that CMF might also play a role in cell aggregation since anti-sense mutants do not aggregate under starvation conditions. This inability could be attributed to cell paralysis. My studies, however, find that CMF mutants have motility characteristics similar to wild-type amoebae. The low cell density conditions used in this study, however, may have masked the effects that chemotaxis plays in cell aggregation and suggests that CMF could have a
role in the chemotactic process, possibly by inducing expression of genes responsible for the establishment and maintenance of cAMP cell surface receptors. Another possibility is that CMF antisense mutants fail to aggregate because they cannot produce adhesion molecules, such as discoidins, that are required for proper aggregation.
V. References


Poole, T. J. and Steinberg, M. S. *Dev. Biol.* 92: 144-158 (1982).


Yuen, S. W., Chui, A. H., Wilson, K. J., and Yuan, P. M. *Biotechniques* 7: 74-83 (1989).

### VI. Appendix A

**Table of Abbreviations**

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<tr>
<td>CMF</td>
<td>conditioned medium factor</td>
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<td>CMF-H</td>
<td>high molecular weight CMF</td>
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<td>CMF-L</td>
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<td>DTT</td>
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<td>HPLC</td>
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<td>kD</td>
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<td>LISB</td>
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VII. Appendix B

Peptide Sequences from *Dictyostelium* Cytoskeleton Preparation.

A major sequence was easily identifiable for each peptide fraction subjected to Edman degradation. Several fractions also contained minor peptide components, which could not be identified separately because they were present in similar quantities. These components are denoted \((X/Z)\) for each cycle, where \(X\) and \(Z\) represent the amino acids.

1) Peptide from 36 kD protein obtained from urea extract of early developmental cells:


   Minor Sequences: \((D/M)-(Q/N)-(M/A)-(D/N)-(Q/E)-(W/K)-(V/Y)-\)
   \((T/H)-(D/S)-(Y/F)-(K/D)-(F/G)-(W/L)-(L/V)\)

2) Peptide from 36 kD protein obtained from urea extract of early developmental cells:

   Major Sequence: \(E-V-V-V-P-V-K-F-Q-F-E-F-A-T\)

   Minor Sequence: \((R/G)-(I/F)-(T/M)-(N/Q)-(R/F)-(R/A)-(Y/Q)-(L/R)-\)
   \((G/A)-(N/L)-(F/E)-(R/N)-(T/V)-(T/H)\)

3) Peptide from 50 kD protein obtained from urea residue of vegetative cells:


   Minor Sequence: \(I-V-H-P-S-Y-N-S-N-T-L-N-N-D-I-M\)