Regulation and processing of a secreted protein that mediates sensing of cell density in Dictyostelium

ITA S. YUEN, CARL TAPHOUSE, KERRI A. HALFANT and RICHARD H. GOMER*

Howard Hughes Medical Institute and Department of Biochemistry and Cell Biology, Rice University, Houston, TX  77251-1892, USA

*To whom correspondence should be addressed

Summary

During Dictyostelium development, the expression of some genes is dependent on cell density. This effect is mediated by soluble factors referred to as conditioned medium factors (CMFs) which the developing cells secrete at very low rates and simultaneously sense. There are at least two classes of CMFs: one is an $80 \times 10^3$ Mr glycoprotein and the other is a heterogeneous group of molecules, with relative molecular masses between $6.5 \times 10^3$ and $0.65 \times 10^3$. Interestingly, the two classes of molecules do not need to be combined for activity. We find that the $80 \times 10^3$ Mr CMF but not the small CMF is sequestered in vegetative cells. The $80 \times 10^3$ Mr CMF is then secreted by cells during early development, while the small CMF appears only during late development. Like the $80 \times 10^3$ Mr CMF, the small CMFs are trypsin-sensitive and contain N- and O-linked glycosylation. The breakdown products of a fraction containing $80 \times 10^3$ Mr CMF cochromatographed from a Sephadex G-50 column and a reverse-phase HPLC column with small CMFs. The specific activity of CMF increases roughly 100-fold upon breakdown. The results suggest that, during differentiation, the slowly diffusing $80 \times 10^3$ Mr CMF is first produced from a precursor pool already present in vegetative cells, allowing differentiation of only those cells in the immediate vicinity of the aggregation center. The breakdown of $80 \times 10^3$ Mr CMF to a faster-diffusing, higher specific activity form then might enable cells farther from the aggregation center to differentiate.

Key words: Dictyostelium, secreted protein, glycopeptide, mass sensor.

Introduction

Very little is known about the mechanisms that control tissue size and density. One way for cells to sense their density is to secrete an autocrine factor that they simultaneously sense. Examples are the C-factor secreted by high density Myxococcus xanthus (Kim and Kaiser, 1990a,b), the autoinducer produced by the light organ symbiont Vibrio fischeri (Eberhard et al. 1981), the extracellular differentiation factor A that stimulates spore formation of Bacillus subtilis (Grossman and Losick, 1988) and the Dictyostelium discoideum conditioned medium factor (Mehdy et al. 1983). In all of these systems, gene expression patterns are affected by the concentration of the secreted factor.

Dictyostelium is one of the simplest systems for the study of density sensing during eukaryotic development. Approximately six hours after starvation, these unicellular soil amoebae stream together using pulses of cAMP as the chemoattractant. The aggregate of typically $10^6$ cells then elongates and forms into a migrating slug or pseudoplasmodium. After migrating to a favorable environment, the slug differentiates into a fruiting body consisting of two cell types: a mass of spore cells supported by a ~2 mm high stalk. The complete developmental cycle takes about 24–26 h (Loomis, 1975, 1982).

10–12 h after starvation, precursors to stalk and spore cells (prestalk and prespore cells, respectively) become spatially organized in the pseudoplasmodium. The prestalk-specific protein, pst-cathepsin (cysteine protease 2), can be detected as early as 10 h after starvation whereas the prespore coat protein, SP70 (beejin), appears at 15 h; pst-cathepsin mRNA appears as early as 7 h and SP70 transcripts as early as 8 h (Barklis and Lodish, 1983; Mehdy et al. 1983; Morrissey et al. 1984; Devine and Loomis, 1985; Mehdy and Firtel, 1985; Datta et al. 1986; Gomer et al. 1986b; Saxe and Firtel, 1986; Wang et al. 1986; Williams, 1988; Haberstroh and Firtel, 1990; Esch and Firtel, 1991). Cell fate is initially determined by cell cycle phase at the time of starvation (Weijer et al. 1984; McDonald, 1986; Gomer and Firtel, 1987). During later development, extracellular factors such as ammonia, adenosine,
oxygen and chlorinated hydrocarbons (DIFs) then modulate the ratio of cell types (Gross et al. 1983; Schaar and Wang, 1986; Brookman et al. 1987; Williams et al. 1987; Sternfeld, 1988; Kwong and Weeks, 1989; Xie et al. 1991).

Extracellular cAMP regulates gene expression at different developmental stages. During aggregation, pulses of cAMP released by starving Dictyostelium cells interact with cell-surface cAMP receptors on neighboring cells. The receptors transiently activate two intracellular signal transduction systems. One system activates adenylate cyclase which eventually leads to amplification and propagation of cAMP signals (Devreotes, 1982; Gerisch, 1987; Janssens and Van Haastert, 1987; Kessin, 1988). The second pathway involves the activation of phospholipase C which finally results in chemotaxis and activation or repression of some developmentally regulated genes (Janssens and Van Haastert, 1987; Mann and Firtel, 1987; Mann et al. 1988). After completion of aggregation, the activation of the second signal transduction pathway by a continuous level of at least 30 nM extracellular cAMP is believed to induce the expression of the prespore- and prestalk-specific genes (Mehdy et al., 1985; Gomer et al. 1986a).

In addition to cAMP, a second extracellular molecule, conditioned medium factor (CMF), is required for the induction of a variety of developmentally regulated genes. Firtel and coworkers (Mehdy et al. 1983; Mehdy and Firtel, 1985) found that by starving a monolayer of submerged Dictyostelium cells at a density lower than 5x10^6 cells cm^-2 (no cell-cell contact), no induction of prespore and prestalk gene expression was observed even if extracellular cAMP was added at high levels 6h after starvation. However, cells at this density responded to extracellular cAMP if they were plated in buffer previously conditioned by starving high density cells (conditioned medium). This indicated that cells at high density secrete a factor (CMF) necessary for prespore and prestalk gene expression. A proposed function of CMF is that during development, Dictyostelium cells use CMF to monitor cell density in order to determine whether they are far from an aggregation center and thus need to continue expressing aggregation-specific genes, or are at or near an aggregation center and can thus begin expressing cell-type-specific genes. Similar effects using different assay conditions have also suggested the existence of a molecule such as CMF during development (Grabel and Loomis, 1976) and during vegetative growth before starvation (Clarke et al. 1987, 1988).

Purification and characterization of CMF indicates that two size classes of molecules secreted by developing Dictyostelium cells contain the ability to induce prespore and prestalk gene expression. The high relative molecular mass conditioned medium factor has been purified to an 80x10^6 M_r, glycoprotein, which is highly unstable (Gomer et al. 1991). In this report, we examine the regulation and processing of the low relative molecular mass CMF.

Materials and methods

Cell culture

Axenically growing Dictyostelium discoideum strain KA-x-3 was used for all experiments. Cells were maintained in HL-5 (Firtel and Bonner, 1972) with the following modifications in the recipe: 200 μl of a mixture of ampicillin (10 mg ml^-1), tetracycline (25 mg ml^-1), and chloramphenicol (25 mg ml^-1) and 0.8 ml of 500 g l^-1 glucose were added to 20 ml of a mixture of 14.3 g l^-1 peptone (Oxoid Limited, Basingstoke, Hampshire, England), 7.15 g l^-1 yeast extract (Oxoid), 0.4 g l^-1 Na_2HPO_4, 0.2 g l^-1 NaH_2PO_4, and 0.55 g l^-1 KH_2PO_4. For conditioned medium (CM) production, mid-logarithmic cells (<5x10^6) were used whereas for the CM assay, cells growing at a density of less than 1x10^6 cells ml^-1 were used. Conditioned medium was made by centrifuging cells at 500 g for 5 min, resuspending cells in PBM (20 mM KH_2PO_4, 1 mM MgCl_2, 0.01 mM CaCl_2, pH 6.1 with KOH), refrigerating, resuspending to 5x10^6 ml^-1, and shaking at 110 revs min^-1 for 20 h.

Conditioned medium, CMF preparation and conditioned medium assay

CMF-containing conditioned medium and purified CMF were produced as described (Gomer et al. 1991). Conditioned medium activity was determined using a modification of an immunofluorescence assay (Gomer et al. 1986a,b) to measure the expression of prespore-specific genes by developing low density Dictyostelium cells. The quantitation of the prespore antigen SP 70 by immunofluorescence has been described in detail (Gomer et al. 1991). It is important to note that cells expressing SP 70 in this assay arise from only one of each pair of sister cells that happened to be in a ~6h quadrant of the ~8h cell cycle at the time of starvation; thus the maximum number of SP 70-positive cells is 1/2 (6/8)=37.5% (Gomer and Firtel, 1987). As previously defined (Gomer et al. 1991), CMF activity is expressed as the dilution of material at which the number of cells induced to express SP 70 falls to 50% of its maximum; thus 100 units ml^-1 of activity indicates that at a 100-fold dilution, the number of SP 70-positive cells is half of the attainable number in that assay of SP 70-positive cells (typically 30%). The expression of SP 70 was also quantitated by ELISA. A 96-well microtiter plate (Corning type 4818, Corning, NY) was used with all volumes reduced by a factor of four relative to the eight-chamber glass slides. Higher cell number per area (2.7x10^6 cells cm^-2) was also required so that after the second wash in PBM, cells were resuspended to a density of 4x10^6 cells ml^-1. The plate was also allowed to air dry for an hour and preblocked first with 30 mg ml^-1 BSA for 30 min and then with 10% Carnation nonfat milk in TBS (TBST without Tween-20). The entire well was preblocked to reduce any nonspecific binding of antibodies. Anti-SP 70 antibody was used as the first antibody and goat anti-rabbit IgG antibody conjugated to alkaline phosphatase (Sigma, St Louis, MO) was used as the second antibody. Phosphatase substrate (Sigma) was prepared freshly each time according to the manufacturer's instruction and 50 μl was added to each well. The reaction was carried out at room temperature until a detectable yellow color developed and was stopped by the addition of 50 μl of 1 M potassium phosphate, pH 10.5 and 30 μl of 1 M NaOH. The absorbance was read on an automated microplate plate reader (Perkin-Elmer Lambda Reader, Norwalk, CT) at 405 nm. Since the reaction times for the alkaline phosphatase-conjugated second antibody and its substrate were not fixed, absorbance at 405 nm should only be
Fig. 1. Crude plasma membranes from vegetative cells were electrophoresed on an SDS–polyacrylamide gel. The gel was rinsed briefly and cut into 0.25 cm slices. Material was eluted from the slices, and a series of dilutions were assayed for CMF activity. Quantifiable activity was found in only one slice (bar). Relative molecular masses were determined by running standards in an adjacent lane, staining and correcting for shrinkage. 0 cm represents the bottom of the stacking gel and the dye front was at 6.9 cm.

used for qualitative comparison. Triplicates were used for each sample when ELISA was performed.

Plasma membrane isolation from 1 × 10^9 cells was carried out using the procedure of Das and Henderson (1983) as described by Goodloe-Holland and Luna (1987). Briefly, cells were lysed by passage through a 5 μm pore size filter. The lysate was centrifuged, and the membrane-containing pellet was layered on a 0.75 to 1.5 M sucrose gradient in 50 mM glycine, pH 8.5 which was then centrifuged at 4°C for 18 h at 145,000 g. The supernatant from the first centrifugation, all the visible membrane bands from the sucrose gradient and the sucrose gradient pellet were assayed for CMF activity at dilutions of 1:10, 1:100, 1:10^3, and 1:10^4 using the method described above. A Fisher (Pittsburgh, PA) hand refractometer was used to measure the sucrose concentrations of a set of standard concentrations in the above buffer and the sucrose concentration of the CMF-containing band. Ten percent of the band containing CMF activity was separated on a SDS–10% polyacrylamide gel alongside a lane containing relative molecular mass standards. The lane containing the relative molecular mass standards was stained with Coomassie blue and used to construct a plot of log (relative molecular mass) vs. migration distance (Fig. 1). The lane containing the membrane sample was soaked in distilled water for 5 min to remove SDS and then cut into 2.5 mm sections which were eluted into 1 ml of PBM overnight at 12°C. The relative molecular mass range of the section containing CMF activity was determined by comparison with relative molecular mass markers. The concanavalin A (Con A) stabilization plasma membrane purification procedure (Goodloe-Holland and Luna, 1987) was done as described with the exception that 5.8 × 10^9 cells were used, and volumes were reduced accordingly.

Size fractionation

Whole conditioned medium was size fractionated by one of three methods. In the first method, it was pressure dialyzed against a PTGC 10 × 10^3 M, cutoff membrane (Millipore, Bedford, MA) using a Millipore minitan pressure dialyzer at a back pressure of 25 psi. The material retained by the membrane contained the high relative molecular mass conditioned medium factor (CMF-H), and the filtered material contained the low relative molecular mass conditioned medium factor (CMF-L). In the second method, 1 ml of CM or a CMF-containing fraction was loaded on a 26 cm high × 0.7 cm diameter column packed with Sephadex superfine G-50 resin (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Elution was done at room temperature with PBM at a flow rate of 0.2 ml min^-1 and 1.1 ml fractions were collected. Each fraction was tested for CMF activity. Relative molecular mass standards (blue dextran, 2000 × 10^3; BSA, 66 × 10^3; aprotinin, 6.5 × 10^3; and bromphenol blue, 0.67 × 10^3) were also loaded onto the column for calibration before and after sizing the CMF. The third method to separate CMF-H and CMF-L was by centrifugation of a 400 μl sample in a Millipore Ultrafree-MC ultrafiltration unit with a relative molecular mass cutoff of 10 × 10^3. Centrifugation was done at 2000 g for 45 min at 4°C or until >99% of a sample was spun down. The >10 × 10^3 M fraction was resuspended in PBM to a final volume of 400 μl so that the concentrations for the large molecules would not be changed. The spin-through contained CMF-L and molecules smaller than 10 × 10^3 M.

To examine the kinetics of CMF-H and CMF-L secretion, log phase cells were centrifuged and washed twice in PBM. They were then resuspended in PBM to a density of 5 × 10^6 cells ml^-1. This point was considered to be the beginning of development (t = 0h). Conditioned medium was made as described except that at t = 10 h, 1 ml of the medium was removed and clarified of cells as described above. CMF-H and CMF-L fractions were separated immediately at each time point by centrifugation of 400 μl of conditioned medium through a 10 × 10^3 M, cutoff Millipore Ultrafree-MC ultrafiltration unit for 45 min or until 99% of the liquid was spun through. PBM was added to the retained material (>10 × 10^3 M) to a final volume of 400 μl. The separated CMF-H and CMF-L fractions were stored at −20°C. After thawing, CM assays were done using serial factor of 2 dilutions for >10 × 10^3 M fractions and serial factor of 3 dilutions for <10 × 10^3 M fractions.

Trypsinization of CMF-L

Whole conditioned medium was size fractionated by centrifugal ultrafiltration. TPCK-treated trypsin (Sigma) in PBM was preincubated in the presence and absence of soybean trypsin inhibitor (Sigma) for 5 min before the addition of 300 μl of CMF-L. The final concentrations of trypsin and its inhibitor were each 1 mg ml^-1. The reaction was carried out at room temperature for two hours. At the end of the incubation time, trypsin digestion was terminated by the addition of soybean trypsin inhibitor (STI) to 1 mg ml^-1 and conditioned medium activity was measured using serial factor of 3 dilutions.

Peptide chromatography

One liter of the CMF-L-containing fraction from pressure dialysis of conditioned medium was lyophilized, resuspended in 9 ml of water and dialyzed against water in a 1 × 10^3 M, cutoff Spectra/Por 6 membrane (Spectrum, Los Angeles, CA). After clarification by centrifugation at 24,000 g for 5 min, this was loaded on a 0.7 ml bed volume Dowex 50W-X8 column (Bio-Rad Laboratories, Richmond, CA) in a 1 ml syringe with a glass wool plug, and eluted stepwise with 2 ml each of 0, 0.1, 0.3, and 0.6 M NaCl in PBM at room temperature. Fractions eluting from the column were assayed for CMF activity. A 0.6 ml bed volume Con-A column
The spin-through fractions were assayed for CMF activity on saccharides at the \( \alpha\)-aspartyl-glucosylamine bond. Neuraminidase digestion was carried out using whole CM substrates centrifuged through Millipore ultrafiltration units as described. Reactions were terminated by separating the enzymes from the O-glycanase (1 mU) at room temperature for 16 h. The O-glycanase (1 ml; Genzyme), neuraminidase (20 mU; Genzyme), endoglycosidase H (1 mU; Boehringer Mannheim), and N-glycanase (250 mU; Genezyme Corporation, Boston, MA) were used. A 10 x 100 mm column of Sephadex G-25 (Pharmacia) was equilibrated in 0.5% acetic acid, 1 ml fractions were collected and every fraction was assayed for CMF activity.

1 ml of fractions 18 and 30 from the size fractionation experiment were loaded individually on a Dynamax 5 mm particle size, 4.6 x 250 mm HPLC C8 column (Rainin, Emeryville, CA) and eluted with a linear gradient of 0.1% trifluoroacetic acid (TFA) in water to 0.08% TFA in acetonitrile over a period of 60 min at a flow rate of 1 ml per minute. Absorbance was read at a wavelength of 214 nm and 1 ml fractions were collected. Because of the presence of TFA, HPLC fractions to be assayed for CMF activity were dried by Speed-vac (Savant Instruments, Inc., Farmingdale, NY), resuspended in 1 ml of water, redried and finally resuspended in 1 ml of PBM. Peaks of CMF activity from the Sephadex column were also loaded on a 4.6 x 250 mm C18 reverse phase HPLC column (Custom LC Inc., Houston, TX) and a 4.6 x 250 mm HPLC phenyl column (Vydac, Hesperia, CA).

Deglycosylation

Various endoglycosylases were used to determine the effect of deglycosylation on CMF activity. As summarized in the data sheets from the suppliers listed below, endoglycosidase F and H specifically hydrolyze high mannose N-glycans leaving one molecule of N-acetylglucosamine bound to asparagine, N-glycanase catalyzes hydrolysis of asparagine-linked oligosaccharides at the \( \beta\)-aspartyl-glucosylamine bond, neuraminidase removes \( \alpha\)-Gal, \( \alpha\)-GalNAc core disaccharide attached to serine or threonine residues of glycoproteins; any substitutions (e.g. sialic acid) will inhibit cleavage. CMF-L was produced by centrifuging whole CM substrates using Millipore ultrafiltration units as described. The spin-through fractions were assayed for CMF activity using a series of 3-fold dilutions. To examine the glycosylation of CMF-H, 16 \( \mu\)l of DEAE and hydroxylapatite-purified CMF-H (Gomer et al. 1991) was mixed with 1.6 \( \mu\)l of 5x Laemmli sample buffer, 1.6 \( \mu\)l of 200 mM EDTA and 52.8 \( \mu\)l of water. After boiling for 2 min, 8 \( \mu\)l of 10% NP-40 and 1 \( \mu\)l of 50 mM PMSF, 50 mM TAME, 62 mM O-phenanthroline and 25 mM benzamidine were added to the mixture. 1 \( \mu\)l of the enzymes as described above (or 2 \( \mu\)l of the neuraminidase/O-glycanase mix) was then added to 10 \( \mu\)l aliquots of the CMF-H/detergent mixture and incubated at 37°C for 11 h and 15 min. 5 \( \mu\)l of the digest mixture was mixed with 15 \( \mu\)l of 1xLaemmli sample buffer and electrophoresed on an SDS-polyacrylamide gel that was then silver stained as described (Gomer et al. 1991).

Results

Density sensing factor is associated with the membrane of vegetative cells

Measurements of conditioned medium factor (CMF) production at different developmental stages showed that CMF is released from Dictyostelium cells during the first five hours of development and also throughout later development (Mehdy and Firtel, 1985) but not from vegetative cells (Gomer et al. 1991). To determine if the appearance of CMF during early development arises from de novo synthesis, or if this early appearance of CMF involves release of CMF sequenced in vegetative cells, we examined subcellular fractions of vegetative cells for CMF activity.

Cells were fractionated by the method of Das and Henderson (1983) as described by Goodloe-Holland and Luna (1987). This yields two separable sucrose gradient bands containing plasma membranes and other subcellular membranes. One of the bands, which Goodloe-Holland and Luna designated the 'combined 1 and 2 bands', showed significant levels of CMF activity; this band was at a sucrose concentration of 1.28 M. No CMF activity was in the first supernatant (crude cytosol), or in any other band or the pellet from the sucrose gradient. An SDS-polyacrylamide gel of this band was stained with Coomassie, and showed a protein profile that was essentially identical to the protein profile of combined bands 1 and 2 shown in Fig. 5 of Goodloe-Holland and Luna (1987). In contrast, a Con A-stabilization, Triton-extraction procedure used to purify plasma membrane (Goodloe-Holland and Luna, 1987) failed to show any significant levels of CMF activity in the plasma membrane fraction.

A sample of the sucrose gradient band containing the CMF activity was electrophoresed on an SDS-polyacrylamide gel. Material eluted from slices of the gel was assayed for CMF activity as described (Gomer et al. 1991). As shown in Fig. 1, the only slice containing CMF activity was one that contained proteins with relative molecular masses between 84 and 91 x 10^3, roughly corresponding to the relative molecular mass of CMF-H (Gomer et al. 1991). The above observations suggest that vegetative cells contain a membrane-associated 84 to 91 x 10^3, protein that has CMF activity.

CMF-H is secreted earlier than CMF-L

Mehdy and Firtel (1985) observed that for cells
developed at an air–water interface, CMF activity is secreted throughout development, with a higher rate during aggregation stage. To determine if CMF-L is secreted throughout development, CMF-L and CMF-H activities were separately measured for conditioned medium collected at 10 h and 20 h of starvation. The >10 × 10^3 M_r fraction of 10 h conditioned medium contained 5 units ml^-1 of activity and the >10 × 10^3 M_r fraction of 20 h conditioned medium contained 7 units ml^-1 of activity (Table 1). CMF-L activity was not detected in 10 h conditioned medium; at 20 h, CMF-L activity was only 3 units ml^-1 (Table 1).

**Table 1.**

<table>
<thead>
<tr>
<th>Hour after starvation</th>
<th>Activity (units ml^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&gt;10 × 10^3 M_r fraction</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>20</td>
<td>7</td>
</tr>
</tbody>
</table>

Time courses for secretion of CMF-H and CMF-L. Cells were starved in PBM at a density of 5 × 10^6 cells ml^-1 for 10 or 20 h. After clarification, conditioned media made for the different time periods were centrifuged through a Millipore Ultrafree-MC ultrafiltration device to separate materials > and <10 × 10^3 M_r. After bringing the final volume to 400 μl for both fractions, the spin-through material (<10 × 10^3 M_r) and the retained material (>10 × 10^3 M_r) were diluted serially and assayed for CMF activity.

Low relative molecular mass CMFs are polypeptides

CMF-H is a trypsin-sensitive 80 × 10^3 M_r glycoprotein which tends to break down to a 65 × 10^3 M_r protein during the process of being eluted from an SDS–polyacrylamide gel, whereas CMF-L is a set of molecules with relative molecular masses less than 10 × 10^3 (Gomer et al. 1991). To determine if CMF-L is also trypsin-sensitive, a fraction containing CMF-L was treated with either trypsin, trypsin and soybean trypsin inhibitor (STI), or STI alone. At the end of a 2 h room temperature incubation, soybean trypsin inhibitor (STI) was added to the reaction containing trypsin. The samples were then assayed for CMF activity. When CMF-L was treated with trypsin for 2 h, CMF activity was destroyed, suggesting that CMF-L is a polypeptide (Table 2). However, if trypsin was inhibited with STI during the reaction time, CMF activity could still be detected (Table 2). Interestingly, the presence of STI actually enhanced the activity of CMF 8-fold.

80 × 10^3 M_r, CMF and CMF-L have similar types of glycosylation

We have previously shown that CMF-H binds to the lectin concanavalin A (Con-A) indicating that it contains high mannose oligosaccharides (Gomer et al. 1991). To characterize further the types of oligosaccharide groups associated with the 80 × 10^3 M_r CMF, the DEAE and hydroxyapatite-purified CMF-H containing fraction was prepared as described (Gomer et al. 1991) and treated with various deglycosylating enzymes or water before electrophoresis on an SDS–polyacrylamide gel. To monitor the degradation of the 80 × 10^3 M_r CMF, an equal amount of the untreated fraction was also electrophoresed (Fig. 2, lane Fr). As previously described (Gomer et al. 1991), the 80 × 10^3 M_r CMF partially degraded at room temperature (compare Fig. 2, lanes Fr and W). As seen in Fig. 2, treatment of the CMF-H-containing hydroxylapatite fraction with endoglycosidases F and H or N-glycanase (lanes F, H and G) resulted in the disappearance of the 80 × 10^3 M_r protein band (arrow, Fig. 2), indicating that CMF-H contains high mannose glycosylation.

**Table 2.**

<table>
<thead>
<tr>
<th>Trypsin</th>
<th>STI</th>
<th>CMF activity (units ml^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>6</td>
</tr>
<tr>
<td>None</td>
<td>2 h</td>
<td>50</td>
</tr>
<tr>
<td>0 h</td>
<td>2 h</td>
<td>0</td>
</tr>
<tr>
<td>0 h</td>
<td>0 h</td>
<td>45</td>
</tr>
<tr>
<td>0 h (boiled)</td>
<td>None</td>
<td>7</td>
</tr>
</tbody>
</table>

Effect of trypsin and/or soybean trypsin inhibitor (STI) on CMF-L activity. CMF-L was isolated from whole conditioned medium as described. Ten units of CMF-L was treated with trypsin and/or trypsin inhibitor (final concentration of 1 mg ml^-1, respectively) at the time indicated for 2 h at room temperature. At the end of the reaction time, CMF activity was determined as described using serial factor of 3 dilutions.

Fig. 2. The 80 × 10^3 M_r CMF has both N- and O-linked glycosylation. A column fraction of partially purified 80 × 10^3 M_r CMF was divided into 8 aliquots. Seven of the aliquots were treated with enzymes or water overnight. Samples were then electrophoresed on an SDS–polyacrylamide gel which was then silver stained. Lane M, relative molecular mass markers; the relative molecular masses × 10^-3 are indicated. Lane Fr is an aliquot of the crude 80 × 10^3 M_r CMF that did not sit out overnight. Overnight digestions were F, endoglycosidase F; H, endoglycosidase H; G, N-glycanase; N, neuraminidase; N/O, neuraminidase and O-glycanase combined; O, O-glycanase; W, water alone.
There are multiple CMF-Ls that do not bind to concanavalin A. The flow through fractions from Con-A chromatography of CMF-L (did not bind to Con-A) were loaded onto a Sephadex G-25 column to separate the CMF-L by size. The column was run at room temperature in 0.5% acetic acid. A bar above a fraction indicates that the fraction contained measurable CMF activity. Four peaks of CMF activity were observed, indicating that the CMF-L is a family of peptides of varying sizes.

To determine whether CMF-L is also glycosylated, a concentrated $1 \times 10^3 M_r$ fraction of conditioned medium (approximately 16 ml of material with an OD 280 of 1.1) was chromatographed on a Con-A column. The flow-through fractions were then fractionated on a Sephadex G-25 column; four peaks of CMF activity were observed (Fig. 3). The fraction eluted from the Con-A column by methyl $\alpha$-D-mannopyranoside (presumably containing glycosylated peptides) was similarly chromatographed, which revealed three peaks of CMF activity (Fig. 4). CMF activity was also observed in both the flow through and the sugar-eluted fraction when the same Con-A column was loaded with 8 ml of unconcentrated material (OD 280 = 0.01). This suggests that the observation that some CMF-L binds to Con-A and is eluted by methyl $\alpha$-D-mannopyranoside, and some CMF-L does not bind to Con A, is not due to column overloading. These results indicate that CMF-L consists of a heterogeneous group of molecules, some of which contain high mannose oligosaccharide, and others of which, although possibly glycosylated, do not contain high mannose oligosaccharide.

Since there is heterogeneity in the glycosylation of CMF-L, we examined whether the sugar moieties are important for CMF activity. CMF-L was incubated with endoglycosidases F or H, N- or O-glycanase, neuraminidase, a combination of neuraminidase and O-glycanase, or water. As shown in Table 3, removal of sugar moieties by any of the above enzymes greatly reduced the activity of CMF-L, indicating that CMF-L contains...
glycosylations that are cleaved by the above enzymes and thus these glycosylations are important for its activity. The removal of sialic acid from CMF-L by neuraminidase did not reduce CMF activity as drastically as the other deglycosylation enzymes. Thus CMF-L, like the $80 \times 10^3 M_r$ CMF, appears to contain both N- and O-linked glycosylation.

**Low relative molecular mass CMFs may originate from the $80 \times 10^3 M_r$ CMF**

The results described above indicate that CMF-H and CMF-L are both glycosylated polypeptides. To test whether the CMF-Ls originated from the $80 \times 10^3 M_r$ CMF, whole conditioned medium was size fractionated on a Sephadex superfine G-50 column. Fractions were assayed with ELISA. As observed previously, one peak of activity (fraction 8) coeluted with blue dextran and BSA whereas multiple peaks are seen having relative molecular masses smaller than aprotinin ($6.5 \times 10^3 M_r$) (Fig. 5A). CMF activity can be detected in fractions eluted after bromphenol blue ($0.65 \times 10^3 M_r$) presumably due to nonspecific interactions between some conditioned medium factors and the Sephadex resin, as has been observed for other polypeptides (Savage and Cohen, 1972). When fraction 8 was immediately refractionated by the same column, only one peak of activity was observed, coeluting again with blue dextran and BSA (Fig. 5B). In order to detect low relative molecular mass breakdown products of CMF-H, fraction 8 was allowed to sit out at room temperature for 24 h and was then rechromatographed. An increased number of peaks of activity were observed, some of which coeluted with CMF-L from whole conditioned medium (Fig. 5C). This suggested that there were molecules of the same sizes found both in conditioned medium and the breakdown products of CMF-H. Incubation at room temperature for 72 h resulted in the disappearance of the activity peak at fraction 8 (data not shown). However, peaks of activity with relative molecular masses smaller than $\sim 6.5 \times 10^3$ remained.

Fraction 31 from the G-50 chromatography of whole conditioned medium and fraction 31 from the G-50 chromatography of the breakdown products (CMF-H sat out at room temperature for 24 h) consistently contained conditioned medium activity. To compare further the CMF-L from whole conditioned medium and the degraded CMF-H, the two fraction 31s were rechromatographed individually using C8 reverse-phase HPLC (Fig. 6). Each peak of absorbance at 214 nm and the two fractions on either side of it were assayed for CMF activity. Comparing the elution profiles of the two samples, at least seven peaks from the whole conditioned medium sample (Fig. 6A) eluted at the same acetonitrile concentrations as did peaks from the degraded CMF-H sample (Fig. 6B). Only two peaks from whole CM had CMF activity (*, Fig. 6A), degraded CMF-H also had only two peaks (*, Fig. 6B). In both samples, the two CMF activity peaks eluted at 24% and 35% acetonitrile, indicating that CMF-L polypeptides with the same Sephadex superfine G-50
As seen in Fig. 5, there are at least two peaks of CMF activity with relative molecular mass less than \( \sim 6.5 \times 10^3 \). A Sephadex G-50 column equilibrated and eluted with 0.5% acetic acid also showed multiple peaks of CMF activity below \( 6.5 \times 10^3 M_c \). HPLC C8, C18 and phenyl columns also yield multiple active peaks, reinforcing the idea that CMF-L is a family of peptides. CMF activity is not retained on a Dowex 50W-X8 cation exchange column equilibrated in PBM, pH 6, indicating that CMF-L has an acidic isoelectric point.

**Breakdown of CMF-H increases its specific activity**

Half of the CMF-H-containing fraction 8 from a G-50 Sephadex column was frozen immediately and the other half was left out at room temperature for 35 h. At the end of the incubation period, the sample was frozen. The two samples were then thawed and CMF activity was measured. The fresh-frozen CMF-H had an activity of 7.3 units ml\(^{-1}\) (Fig. 7A), while the breakdown of CMF-H surprisingly increased the activity to 730 units ml\(^{-1}\) (Fig. 7A). The activity of the fresh-frozen fraction 8 at dilutions below 1:50 dilution is variable from experiment to experiment and may be due to a variable amount of breakdown during the assay. The peak of activity in the degraded CMF between dilutions of 1:50 and 1:5000 is extremely reproducible and appears to represent an increase in the specific activity of CMF upon degradation. To analyze further the composition of the degraded CMF, it was fractionated by ultrafiltration. The two size fractions were then assayed for CMF activity. As seen in Fig. 7B, the majority of CMF activity in the degraded fraction 8 is in the \(<10 \times 10^3 M_c\) fraction. The reduction in the amount of CMF activity after ultrafiltration (compare Fig. 7B with Fig. 7A solid dots) is variable and due to unknown causes. The composition of the frozen fraction 8 was similarly analyzed and found to contain only \(80 \times 10^3 M_c\) CMF activity (data not shown).

**Purified \(80 \times 10^3 M_c\) CMF does not break down to CMF-L by itself**

Fraction 8 from the chromatography of whole conditioned medium on G-50 Sephadex contains, in addition to the \(80 \times 10^3 M_c\) CMF-H protein, of the order of 100 other proteins. To determine if the breakdown of CMF-H to CMF-L is autocatalytic, 12.5 \(\mu\)l of a gel-purified \(80 \times 10^3 M_c\) CMF stock (Gomer et al. 1991) with an activity of 185 units ml\(^{-1}\) was incubated in the presence or absence of 1.6 \(\mu\)l of whole conditioned medium which had an activity of 25 units ml\(^{-1}\). At the end of 72 h, PBM was added to a final volume of 400 \(\mu\)l so that the activity of the gel-purified \(80 \times 10^3 M_c\) CMF was 5.8 units ml\(^{-1}\) whereas that due to the whole conditioned medium was 0.1 unit ml\(^{-1}\) (i.e. the activity from the purified \(80 \times 10^3 M_c\) CMF was 58 times more than that from the whole CM). The two reaction mixtures were then fractionated by ultrafiltration. When the gel-purified \(80 \times 10^3 M_c\) CMF was incubated with whole conditioned medium at room temperature for 72 h, the \(<10 \times 10^3 M_c\) fraction was found to have an


**Discussion**

We have investigated the observation that the *Dictyostelium* conditioned medium factor exists as both an $80 \times 10^3 M_r$ glycoprotein and as much smaller molecules. We find that the conditioned medium factor, which is secreted only after the start of development, can be detected in vegetative cells before development has begun. The factor is associated with a crude plasma membrane fraction from vegetative cells, which suggests that it is trapped inside vesicles or attached to a membrane. The Das and Henderson (1983) crude plasma membrane purification procedure yields membranes distributed in three sucrose gradient bands; the possibility thus exists that the CMF-associated membranes are from some subcellular fraction that are not actually plasma membranes. The lack of CMF activity in the Con-A stabilized plasma membranes may be due to this or to the fact that membranes made by this method lack subdomains of the plasma membranes (Goodloe-Holland and Luna, 1987). Taken together, this suggests that CMF in vegetative cells is either associated with a non-plasma membrane or is associated with a subdomain of the plasma membrane that is not cAPPED by extracellular Con A.

SDS-gel electrophoresis of the crude plasma membrane fraction indicates that most of the CMF activity is associated with an $84 \rightarrow 91 \times 10^3 M_r$ protein; no CMF-L activity was detected. Assuming that 1 unit ml$^{-1}$ represents 0.25 ng ml$^{-1}$ of $80 \times 10^3 M_r$ CMF (Gomer et al., 1991), we can calculate that the 3300 units ml$^{-1}$ material eluted out of the gel slice into 1 ml, given a 25% recovery from a gel slice (from table 2 of Gomer et al., 1991), contained $3.3 \times 10^{-6}$ g of $80 \times 10^3 M_r$ CMF. This represented a purification from one-tenth of $10^9$ cells. The amount of $80 \times 10^3 M_r$ CMF sequestered per cell would then be $3.3 \times 10^{-14}$ g, or $2.3 \times 10^5$ molecules. From the same table 2, there is roughly $(6.7 \times 10^{-5} / 0.32 g)$ of $80 \times 10^3 M_r$ CMF secreted from $(9 \times 10^6 ml ^{-1} \times 2400 ml)$ cells, or $9.7 \times 10^{-16} g (7.3 \times 10^3$ molecules) of $80 \times 10^3 M_r$ CMF secreted per cell over 20 h. Although the actual amount secreted is probably higher because of degradation, oxidation and/or internalization by cells, there appears to be enough sequestered CMF in the vegetative cells to account for all of the observed secretion. We do not know why CMF is sequestered in vegetative cells, nor do we know how much of the secreted CMF arises from the sequestered material and how much is due to CMF synthesized *de novo* during development; however, there exist other proteins that are sequestered in vegetative cells and then are secreted during *Dictyostelium* development (Cardelli et al., 1990).

CMF-L has many similarities to the $80 \times 10^3 M_r$ CMF. Both are protease-sensitive, and appear to contain N-linked glycosylation and O-linked glycosylation. Some of the CMF-Ls appear to have high mannose oligosaccharides. For both CMFs, some of the oligosaccharides are substituted with sialic acid and contain Gal$\beta$(1,3)GalNAc disaccharide as the core. Both impure and SDS-gel-purified $80 \times 10^3 M_r$ CMF...
CMF break down to CMF-Ls. Interestingly, we find that the $80 \times 10^3 M_r$ CMF needs the activity of a protein that elutes in the void volume of a Sephadex G-50 column to be able to break down to CMF-L. We do not know whether the breakdown of the $80 \times 10^3 M_r$ CMF to an active $65 \times 10^3 M_r$ form is autocatalytic or due to protease contamination during the elution from an SDS-polyacrylamide gel (Gomer et al. 1991). The processing of CMF from a larger precursor to a much smaller polypeptide is somewhat similar to the processing of epidermal growth factor (EGF), with the exception that the CMF precursor is soluble whereas the EGF precursor is membrane-bound (Gray et al. 1983; Scott et al. 1983; Mroczkowski et al. 1989). Another difference between CMF-L and EGF is that there are at least seven different CMF-Ls. This could be due either to multiple tandem CMF-L precursors, or to partially cleaved polypeptides that overlap a single active site. Because we use cells that are recloned every month, the heterogeneity of CMF-Ls is probably not due to allelic variation (Smith et al. 1989). Assuming an average CMF-L size of $3 \times 10^3 M_r$, the 100-fold increase of CMF activity upon breakdown requires that, even if there are tandem CMF-L precursors, CMF-L molecules should have a higher molar specific activity than the $80 \times 10^3 M_r$ CMF. Whether the different CMF-Ls have different functions or receptors is unknown. The sensitivity of CMF-L activity to deglycosylation indicates that both the N- and O-linked glycosylations are important for stability of the molecules and/or may be part of the active site.

The biological function of the breakdown of $80 \times 10^3 M_r$ CMF to a molecule that diffuses faster (because it is smaller) and is more efficacious is unclear. Previous experiments have shown that prespore and prestalk gene expression requires exposure of cells to CMF for only the first six hours of starvation, well before the breakdown of CMF occurs. This suggests that CMF-L does not function to maintain expression of these genes. One possibility is that the breakdown allows cells that have not reached the aggregate by the time it is starting to form a fruiting body to express CMF-sensitive genes. Some of these genes, such as discoidin, do not require additional extracellular factors for expression. Others, such as prespore and prestalk genes, require high continuous levels of CAMP, and it is currently unclear whether the isolated cells would be able to express this class of genes.

We wish to thank Kimberly Tanner and Robin Ammann for assistance with the experiments and Bruce Cooper, Rick Firtel, Mike Gustin, Phil Lyons, Fred Rudolph, Tim Spann and Michael Stern for helpful suggestions. This work was supported by NIH grant GM42604, the Howard Hughes Medical Institute and startup funds from Rice University.

References


