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PSF and CMF, autocrine factors that regulate gene expression during growth and early development of *Dictyostelium*

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Abstract. Throughout growth and development, *Dictyostelium* cells secrete autocrine factors that accumulate in proportion to cell density. At sufficient concentration, these factors cause changes in gene expression. Vegetative *Dictyostelium* cells continuously secrete prestarvation factor (PSF). The bacteria upon which the cells feed inhibit their response to PSF, allowing the cells to monitor their own density in relation to that of their food supply. At high PSF/bacteria ratios, which occur during late exponential growth, PSF induces the expression of several genes whose products are needed for cell aggregation. When the food supply has been depleted, PSF production declines, and a second density-sensing pathway is activated. Starving cells secrete conditioned medium factor (CMF), a glycoprotein of Mr 80 kDa that is essential for the development of differentiated cell types. Antisense mutagenesis has shown that cells lacking CMF cannot aggregate, and preliminary data suggest that CMF regulates cAMP signal transduction. Calculations indicate that a mechanism of simultaneously secreting and recognizing a signal molecule, as used by *Dictyostelium* to monitor cell density, could also be used to determine the total number of cells in a tissue.

Key words. Cell density; density sensing; discoidin; prestarvation response; starvation; tissue size.

Introduction

The *Dictyostelium* life cycle embodies several of the features of cellular differentiation in higher organisms, including the use of diffusible molecules as signals for cell-cell communication and regulation of gene expression. Because differentiation occurs relatively synchronously in *Dictyostelium* populations, transitional steps are accessible to biochemical analysis, facilitating the identification of signal molecules.

Soon after the onset of starvation, *Dictyostelium* cells begin to produce one or more small, dialyzable, heat-stable factors that affect gene expression[21,33,39]. A high molecular weight form of one of these (CMF) is described in this chapter. The most intensively studied signal molecule of developing cells is cyclic AMP, which serves as the chemotactic agent during cell aggregation, as a sorting signal for prespore and prestalk cells in the migrating slug, and as a regulator of gene expression (reviewed by Reymond et al. in this volume). A small morphogen, DIF, is involved in stalk-specific differentiation and in establishment of the pattern of cell types in the slug; DIF-1 is a chlorinated phenyl alkanone (reviewed by Berks[2]). The role of these and other diffusible signal molecules in regulating cellular differentiation in *Dictyostelium* has been the subject of several reviews[2,10,58].

Growing cells also communicate by means of autocrine signals. One such signal, DGF, has the properties of a growth factor[57]. Another, PSF, reviewed here, allows cells to monitor their own density in relation to that of their food supply. During late exponential growth, PSF triggers the expression of genes whose products will be needed for cell aggregation. The recognition of this mechanism has given a new perspective on the growth stage of the *Dictyostelium* life cycle. At one level, vegetative cells act as individuals, competing with one another for the bacteria on which they feed. At the same time, they are in communication with each other, collectively and cooperatively keeping track of their population density and anticipating the depletion of their food supply. Thus, even during exponential growth as amoebae, *Dictyostelium* cells are monitoring the parameters that are relevant to the onset of development and are making preparations to become a multicellular unit.

Prestarvation factor (PSF), a density-sensing mechanism of vegetative cells

Initial discovery of the prestarvation response

Earlier studies in other laboratories had shown that amino acid starvation triggers development[37] and that one of the earliest developmentally-induced changes is a dramatic increase in the expression of discoidin-I mRNA and protein (reviewed in Kimmel and Firtel[32]). However, these experiments failed to separate the effects of cell density from those of starvation. Under conditions that made this distinction, it became evident that the synthesis of discoidin-I and certain other early de-

velopmental proteins does not require starvation, but rather is induced by an autocrine factor secreted by growing cells. This *prestarvation response* is triggered in exponentially-growing cells when they reach a certain density relative to that of their bacterial food supply[8,9].

For analysis of the prestarvation response, wild-type *Dictyostelium* amoebae (strain NC4) were grown on a suspension of food bacteria *(Klebsiella aerogenes)* in phosphate buffer. A suspension of bacteria at '1X' concentration supports the exponential growth of *Dictyostelium* cells up to a density of about 1×10^7/ml. Indirect immunofluorescence using an antibody specific for discoidin-I indicated that NC4 cells began to synthesize discoidin-I when they reached a density of about 5×10^5/ml, about four generations before the end of log phase growth. The activity responsible for the induction of discoidin-I synthesis could be detected in the medium in which cells had been growing. This activity was assigned the name 'prestarvation factor', or PSF. Lysosomal enzymes whose activity is elevated in early development (α-mannosidase-1 and β-galactosidase-2) were also up-regulated by this mechanism, suggesting that the prestarvation response affects the expression of a number of proteins previously considered to be induced by starvation[8].

The experiments described above were performed using wild-type (NC4) cells, which grow only on bacteria. Cells of the axenic mutant AX3, which are able to grow on liquid nutrients, were also examined. AX3 cells were known to express a group of early developmental proteins during axenic growth, including discoidin-I and the lysosomal enzymes examined above[1,6]. Discoidin-I expression was monitored as a funciton of cell density in axenically-growing AX3 cells. Very low density cells (4×10^4/ml) produced little or no discoidin-I, while cultures at 1×10^5/ml and higher produced substantial amounts. Thus, discoidin-I appeared in axenic cultures at a much lower cell density than it did in cells growing on bacteria. Axenic growth medium conditioned by high density AX3 cells triggered discoidin-I synthesis in low density wild-type cells. The properties of this inducing activity were indistinguishable from the properties of PSF produced by wild-type cells. These findings suggested that the expression of early developmental genes in axenically-growing cells is regulated at least in part by the prestarvation response[8].

Production of PSF

PSF, assayed by its ability to induce discoidin-I synthesis in low density NC4 cells, was found to be continuously produced by growing cells. Under standard assay conditions, it first became detectable in conditioned buffer as an NC4 culture reached a density of about 5×10^5 cells/ml. However, using pressure filtration to concentrate PSF activity, its presence could also be demonstrated in conditioned buffer from low density cells. The activity present in the medium from an NC4 culture at 5×10^6/ml was comparable to that in the medium of cultures at 5×10^5/ml concentrated tenfold and 1×10^5/ml concentrated fiftyfold[9]. Thus, growing cells continuously produce PSF, which therefore accumulates in the medium in proportion to cell density. Recent studies have shown that the production of PSF declines rapidly when the cells begin to starve, although existing PSF activity persists for several hours[42].

Properties and partial purification of PSF

Although PSF has not been completely purified, its fractionation behavior suggests that it is a glycoprotein of moderate size. PSF activity is destroyed by heat and by proteases. It binds to agarose-conjugated concanavalin-A and can be eluted with α-methyl-D-mannopyranoside. PSF activity present in conditioned buffer can be enriched about twentyfold by ultrafiltration to remove small molecules, followed by affinity chromatography on concanavalin-A agarose (Con-A eluate fraction)[42]. Further purification by gel filtration or ion exchange chromatography leads to large losses in activity. The best results have been obtained using a Sephacryl S300 gel filtration column with buffer containing carrier protein (serum albumin). The elution position of PSF activity from a gel filtration column corresponds to that of a globular protein of about 70 kDa. An additional useful step is absorption with antibodies from a polyclonal antiserum against the lysosomal enzyme α-mannosidase. This antiserum, the gift of Dr. J. A. Cardelli, recognizes a carbohydrate epitope (CA1) that is found on many *Dictyostelium* glycoproteins and is extremely immunogenic[40]. PSF does not bear this epitope. Alpha-mannosidase antibodies conjugated to Protein A-agarose may be used to deplete column-purified PSF of glycoproteins bearing CA1: this removes about 60% of remaining proteins. The final product, partially purified PSF, is approximately one hundredfold enriched in PSF activity relative to the starting conditioned buffer and contains about 3% of the initial activity. This enrichment procedure will be described in greater detail (Burdine and Clarke, submitted for publication).

Inhibition of the prestarvation response by bacteria

Discoidin-I synthesis is a function not only of the *Dictyostelium* cell density but also of the concentration of food bacteria. For a given cell density, the cells produce discoidin-I when the bacterial concentration is low, but not when it is high. When NC4 cells were grown on 1/2 X, 1X, and 2X suspensions of bacteria, discoidin-I synthesis became detectable in each culture about four generations before the end of exponential growth[8]. Similar results were obtained for cells cultured with bacteria on nutrient agar plates[42]. Thus, the cells are able to measure the ratio of their own density relative to that of

their food supply. When the cell density is high and the bacterial concentration is low, the prestarvation response is triggered. Since several gene products induced by the prestarvation response are involved in cell aggregation (see below, next section), this regulatory system allows the cells both to anticipate impending starvation and to determine whether the cell density is high enough to allow aggregation.

The mechanism by which the bacteria exert their inhibitory effect has been investigated. The simplest possibility, that the bacteria destroy PSF activity after it has been secreted into the medium, was found not to be the case. There was no reduction of PSF activity following incubation of conditioned buffer with high concentrations of bacteria, nor was there any inhibitory activity present in buffer that had previously contained bacteria. The possibility that the bacteria block the production of PSF was also tested. Cells were grown on 1/2 X, 1X, and 2X bacterial suspensions until they had reached a density of 3×10^6/ml. At this density, most of the cells in the 1/2 X culture were producing discoidin-I and most of the cells in the 2X culture were not. Conditioned buffer was collected from each of these cultures, used to prepare fresh 1X bacterial suspensions, and inoculated with low density *Dictyostelium* cells. All three samples of conditioned buffer were equally effective at inducing discoidin-I synthesis in low density cells, indicating that they contained equivalent levels of PSF activity. This result indicated that the bacteria do not inhibit production of PSF. Rather, they must interfere with the cells' ability to detect or respond to PSF[9].

Recent studies have shown that heat-killed bacteria are as effective as living bacteria at inhibiting the prestarvation response[5a]. This finding rules out the possibility that the inhibition is due to an unstable, continually replenished bacterial product such as folic acid. These and other data suggest that the inhibition is probably related to the binding of the bacteria to *Dictyostelium* cells.

Role of the prestarvation response in cell aggregation

Several early developmental genes that play key roles at the aggregation stage of development are regulated by PSF. PSF-regulated gene products function in cell streaming (discoidin-I), cAMP signaling (cAMP receptor cAR1 and cyclic nucleotide phosphodiesterase 2.4-kb transcript), cell-cell adhesion (gp24), and lysosome function (α-mannosidase, β-hexosaminidase)[41,42,49]. Particularly interesting is the finding that PSF induces low level expression of the aggregation-specific forms of the cAMP receptor and cyclic nucleotide phosphodiesterase. Earlier work in other laboratories had shown that expression of these genes is induced to high levels by pulses of cAMP[12,14,45], but this mechanism could not account for the initial induction of these genes, since they themselves are needed for cAMP signaling. It now appears that PSF is the regulatory agent that first establishes this signaling system, which then amplifies itself to high levels through a positive feedback loop.

Relationship between PSF and CMF

As described in the second portion of this chapter, at the onset of starvation, *Dictyostelium* cells begin to secrete another glycoprotein that also regulates gene expression in a density-dependent manner. This signal molecule, CMF, is similar to PSF in several respects, although its heat stability and later time of production distinguish it from PSF[19]. However, the discoveries that CMF was actually synthesized by growing cells although not secreted until the cells had begun to starve[60], and that transformants expressing antisense CMF were unable to aggregate[27], raised the possibility that PSF and CMF might be related, perhaps as precursor and product. This possibility was tested by analyzing the production of PSF in AX4 cells transformed with antisense CMF; these transformants had been shown to express negligible levels of CMF mRNA and activity[27]. Their production of PSF activity during growth was compared to that of control cells (untransformed AX4 cells, or AX4 cells transformed with the vector alone) and was found to be essentially normal. Therefore PSF and CMF are distinct gene products[7].

Identification of DNA sequence elements necessary and sufficient for regulation by PSF

Nellen's group has identified three regions upstream of the discoidin-Iγ gene that help to regulate its expression. The method of analysis was to place cloned segments from the region upstream of the discoidin-Iγ gene in front of other (promoter-less) *Dictyostelium* genes or a reporter gene, chloramphenicol acetyltransferase. These studies showed that the 'discoidin induction element' (dIE) was sufficient to confer developmental activation on the reporter gene, and that the essential feature of the dIE was the sequence TTGATTG, called the TTG box[54]. The complementary sequence CAAACAA was also present, implying that the TTG box is an enhancer that can function in either orientation. A second element, the dNCE, was shown to be sufficient for negative regulation by cAMP, and a third element, the dAE, or dAXE, was sufficient for discoidin-I expression during growth on axenic medium[3,54]. Collaborative studies (Nellen and Clarke, unpublished observations) have shown that the element responsible for developmental induction (the dIE) is also sufficient for induction of discoidin-I expression in growing cells exposed to PSF (i.e. conditioned buffer or the Con-A eluate).

The promoter region of a second PSF-regulated gene, that for α-mannosidase[49], has also been analyzed. An element essential for high density axenic or developmental expression was identified, and within this element was a sequence similar to the TTG box of the discoidin-

Iγ gene[48]. Thus, current data, while limited, suggest that the TTG box may prove to be common to all PSF-regulated genes. This promoter element may be the target that lies at the end of the PSF signal transduction pathway. Other elements of this pathway are not yet known. However, heterotrimeric G-proteins do not appear to be involved[5a].

Use of the discoidin-Iγ promoter for inducible expression of cloned DNA sequences

For examination of the role of essential gene products in *Dictyostelium*, it is desirable to use cloning vehicles that allow inducible expression of the cloned DNA sequences. One means of accomplishing this is to regulate the expression of the cloned DNA with a PSF-inducible promoter. An appropriate transformation vector of this type, pVEII, was constructed by Blusch et al.[3]. In pVEII, the discoidin-Iγ promoter is positioned upstream of a multiple cloning site. This promoter has been used to drive the expression of an antisense transcript of calmodulin, a gene presumed to be essential. Cells transformed with this vector were normal in behavior at low cell density, but when they were allowed to grow to high density or were exposed to medium conditioned by high density cells, they lost the ability to complete cytokinesis. Under conditions in which calmodulin protein levels were reduced by about 50%, dividing cells formed a contractile ring that constricted in a normal fashion, but the daughter cells remained connected by a persistent cytoplasmic bridge[35]. This result revealed a previously unrecognized role for calmodulin in cytokinesis.

The discoidin-I promoter has also been used to control overexpression of a truncated transcript of cyclin B. Overexpression of truncated cyclin B caused the cells to arrest in mitosis and led to altered patterns of cell type formation during development[36].

Using a reporter gene to measure promoter activity, Blusch et al.[3] demonstrated that expression from the discoidin-Iγ promoter could be suppressed in high density axenic cultures by the addition of 1 mM folate. This provided an additional means of regulating the expression of antisense or overexpression transcripts driven by this promoter. As described above, it is necessary to maintain axenic cultures at very low cell densities to prevent the expression of discoidin-I. In the presence of 1 mM folate, the threshold for expression is raised about tenfold, so that cells can be grown to densities of 5×10^5 to 1×10^6 cells/ml before discoidin-I protein is detected. This is a more convenient means of manipulating expression for many purposes.

Heterogeneity of the prestarvation response

The prestarvation reponse is often monitored by methods such as Northern blots or enzyme assays that measure the average behavior of an entire population. Such techniques fail to reveal the remarkable heterogeneity of the cellular response to PSF. Figure 1 shows a low density suspension culture of NC4 cells, incubated overnight (i.e. more than five cell generations) in the presence of a moderate concentration of PSF, and immunostained the next morning with antibodies specific for discoidin-I. In the absence of added PSF, these low density cells would all be negative. In the presence of sufficiently high concentrations of PSF, all the cells would be brightly positive. However, at this intermediate PSF concentration, some cells in the population are brightly stained, indicating a high level of discoidin-I protein, others show intermediate levels, and still others are completely negative. The implication of this heterogeneity is that the cells in this clonal population differ greatly in their sensitivity to PSF.

The mechanism responsible for this variation in responsiveness to PSF is unknown. It does not appear to be genetic, because clones isolated from such a population all recapitulate this behavior. Mutations do exist that shift the sensitivity of the population toward one end of the range without eliminating the variability[5a] (also

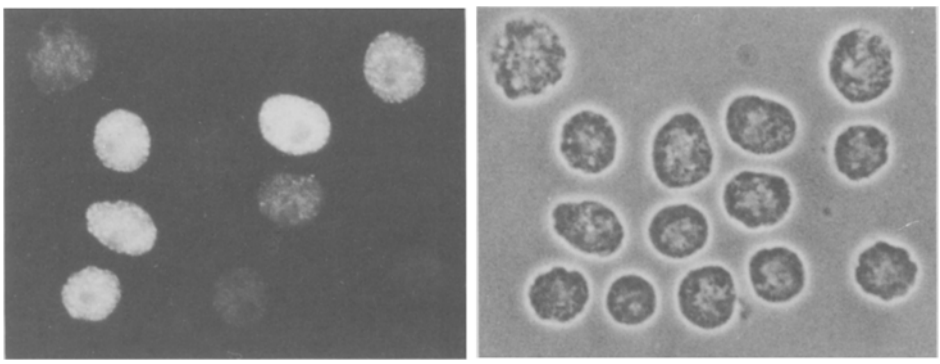

Figure 1. Heterogeneity in cellular response to PSF. NC4 cells growing on a 1X bacterial suspension were incubated overnight with a concentration of PSF sufficient to induce the prestarvation response in about half of the population. Discoidin-I was visualized by indirect immunofluorescence; phase contrast and fluorescence micrographs are shown. Although all cells in this population were exposed to the same concentration of PSF, immunostaining showed that the production of discoidin-I ranged from extremely high to undetectable.

unpublished studies); perhaps these will eventually help to clarify the mechanism. In the meantime, this cell-to-cell variation must be considered when using density or added PSF to induce expression of an antisense or overexpression transcript. The most sensitive cells in the population may respond and die, if the gene is essential, at cell densities or PSF levels that have no effect on the less sensitive cells. Therefore, if possible, it is always desirable to monitor the behavior of individual cells.

Conditioned medium factor (CMF), a density-sensing mechanism of developing cells

Background

Several groups have found that *Dictyostelium* cells starved at low cell densities will differentiate only in buffer conditioned by a high density of cells[17,21]. Using Northern blots to assay for cyclic AMP-induced prespore and prestalk gene expression, Mehdy and Firtel[39] found that cells at relatively high densities (10^5 cells/cm^2) in submerged monolayer culture would differentiate, whereas cells at low density (2×10^3 cells/cm^2) would not. However, when the low density cells were starved in buffer in which high density cells had previously been starved (a conditioned medium; hereafter referred to as CM), the low density cells would then differentiate. At the low densities, the cells are many cell diameters apart and do not contact one another throughout development, as determined by timelapse videotape observations[17]. The differentiation under these conditions indicated that cell-cell contact is not needed for differentiation.

This result suggested that *Dictyostelium* cells secrete a self-stimulating soluble factor (conditioned medium factor, CMF) during development. CMF is secreted throughout development with a slight peak during aggregation[39]. The effect of CMF on the expression of genes that are normally turned on or off during early development is varied. The expression of I42 (a developmentally regulated gene of unknown function) and the lectin discoidin-I, which are both normally expressed only during aggregation, are stimulated by CMF. The expression of vegetative genes such as actin and M4-1, whose expression is repressed during development, is unaffected by CMF[39]. Interestingly, one of the prestalk genes that CMF regulates is *ras*[43,44].

Several other secreted factors which are involved in cellular slime mold development have been described. Differentiation inducing factor (DIF), ammonia and possibly adenosine are all small molecules that have differential effects on prespore and prestalk gene expression[5,29,46,50,55]. Each of these molecules alters the ratio of prespore to prestalk cells within an aggregate. None of these molecules will substitute for CMF (Mehdy, Gomer and Firtel, unpublished observations). D-factor is a small hydrophobic molecule that causes development of non-aggregating mutants of a different slime mold species[23,24], and there exists a small molecule that is required for the accumulation of a developmentally regulated enzyme in *Dictyostelium*[21]. The relationship of the latter two molecules to CMF is unknown. Similar factors have also been observed in bacteria. C-factor, a 17 kDa protein, mediates cell density sensing in starved *Myxococcus xanthus*[30,31]. In *Bacillus subtilis*, the EDF-A protein is secreted by high density cells and is required for cells at low densities to sporulate as efficiently as cells at high densities[22].

The 80 kDa CMF is a novel protein

Because CMF, like PSF, may be a paradigm for the ligand in a cell-density sensing mechanism, we began investigating its properties. We used the fact that cells starved at low density require the presence of CMF for prespore gene expression as the basis for a small-scale bioassay for CMF. To assay a sample (enzyme-treated conditioned medium, conditioned medium from a mutant, or a column fraction) for the presence of CMF, cells were starved at low cell density in submerged monolayer culture (2500 cells/cm^2) in the presence of various dilutions of the sample in the wells of a 96-well microtiter plate or 8-well slide. Prespore and prestalk gene expression requires cells to be exposed to CMF during early development and then to a continuous level of cAMP during later development, so cAMP was added to the wells 6 hours after starvation. The cells were then fixed at 18 hours after starvation and stained for prespore gene expression by indirect immunofluorescence. The staining of prespore antigens would thus indicate the presence of biologically active CMF in the sample. Using this assay, we first found that CMF was protease-sensitive. Gel filtration chromatography indicated that there were two size classes of CMF which did not need to be combined for activity. The first could be partially purified to an 80 kDa glycoprotein while the second size class appeared to consist of 0.5 to 5 kDa molecules; these small CMFs turned out to be breakdown fragments of the 80 kDa CMF (see below). We completed a purification scheme and isolated the 80 kDa CMF[19]. We then sequenced four tryptic peptides of CMF (the amino terminus is blocked) and made a degenerate oligonucleotide probe to screen a *Dictyostelium* cDNA library. A 1.4 kb cDNA fragment was identified that contained a single large open reading frame which had the sequence of three of the tryptic peptides embedded in it[27]. We rescreened a cDNA library to obtain the complete 3′ end of the cDNA, and RACE-PCR was then used to obtain both the 5′ and 3′ ends of the cDNA[26,27]; the sequence of the fourth peptide was found in the additional portion of the large open reading frame from the 3′ region of the cDNA. The complete derived amino acid sequence of CMF

indicated that the polypeptide has a molecular weight of 63 kDa; this is glycosylated to become the 80 kDa CMF (see below). Database searches suggest that CMF has no obvious similarity to any known protein (the latest search was in October 1995). When the cDNA was expressed in bacteria, the resulting fusion protein had CMF activity at 0.3 ng/ml[25], happily similar to the 0.3 ng/ml activity level of the purified native protein. This indicated that the cDNA encodes CMF. In agreement with results from column chromatography of the native CMF protein, the predicted protein is acidic and hydrophilic; the amino acid sequence shows no obvious large regions of charge or hydrophobicity, no unusual α helix or β sheet content, and no other motifs with the exception of an internal signal sequence. Genomic Southern blots indicate that there is a single CMF gene. The sequence data thus indicate that CMF may be a new class of protein signal which mediates density sensing.

A subregion of CMF has full CMF activity

Using deglycosylating enzymes, we found that both the 80 kDa and the small CMFs have N- and O-linked glycosylation and that the small CMFs show greatly decreased activity upon deglycosylation[60]. The CMF cDNA which we expressed in bacteria and which has CMF activity not only lacks the considerable amount of glycosylation that native CMF has, but also represents a 465 amino acid subdomain of the 571 amino acid CMF polypeptide. To delineate the active site of CMF further, we expressed subregions of CMF in bacteria and assayed the resulting fusion proteins for CMF activity. We found that an 88 amino acid region beginning at position 101 had roughly the same molar specific activity as native or the large recombinant CMF. No other region examined had CMF activity[25]. Unlike the native CMF, which is stable for days at room temperature, the recombinant CMFs, especially the 88 amino acid subregion, are labile when in the presence of proteases secreted by starving *Dictyostelium* cells (Jain and Gomer, unpublished). This suggests that the non-active regions of the CMF polypeptide and the N- and O-linked glycosylation of native CMF may protect the CMF active site from proteolysis.

The secretion of 80 kDa CMF is regulated

From the purification of CMF and from examining the amount of CMF secreted after different times of starvation[19,39,60] we were able to calculate that a starved cell secretes roughly 12 molecules of CMF per minute during the first 10 hours of starvation, and slightly lower amounts from 10 to 20 hours after starvation. Northern blots probed with the CMF cDNA indicated that the CMF mRNA is present in vegetative cells and in cells starved for up to 5 hours and is not detectable at or after 7.5 hours of starvation[27]. We had observed that CMF activity cannot be detected in or purified from growth medium conditioned by vegetative cells[19]. It was thus puzzling why vegetative cells contain CMF mRNA but do not secrete CMF, and that cells starved for 10 hours should secrete CMF but do not contain the CMF mRNA. To determine if CMF is sequestered, cells were lysed and fractionated using the procedure of Das and Henderson[11] as described by Goodloe-Holland and Luna[20]. CMF activity was detected only in a crude plasma membrane fraction from both vegetative and 15 hour developing cells[60]. This fraction from the vegetative cells was then mixed with sample buffer without boiling and electrophoresed on a gel. CMF activity could be detected in the material eluted from a slice than contained 84 to 91 kDa proteins[60]. We were able to calculate that the amount sequestered per cell is roughly 2.3×10^5 molecules of 80 kDa CMF, well above the roughly 7.3×10^3 molecules secreted by a cell in 20 hours[19]. To determine the subcellular location of CMF, we used a bacterially synthesized fusion protein consisting of glutathione S-transferase and the central 80% of CMF to immunize a rabbit. The polyclonal serum was affinity purified against a bacterially synthesized fusion protein consisting of maltose-binding protein and the same region of CMF. The antibodies stain 80 kDa CMF on Western blots of conditioned starvation buffer; no CMF is detected in conditioned growth medium. Western blots of vegetative cells indicate that the sequestered CMF has no apparent molecular weight of 85 kDa, suggesting that the secretion involves some sort of processing. In collaboration with Dr. James A. Cardelli and Dr. John Bush (Louisiana State Medical Center at Shreveport), we have found using immunofluorescence that vegetative cells have a combination of punctate and diffuse staining while developing cells show only diffuse staining (Jain et al., submitted for publication). Percoll gradients and trypsinization experiments indicate that some CMF is in the cytosol while some CMF is inside vesicles which fractionate unlike any known organelle in *Dictyostelium*. We conclude that CMF is sequestered in vegetative cells and is then slowly secreted upon starvation.

After development is virtually completed, 80 kDa CMF breaks down to polypeptides with higher specific activities

One of the puzzling aspects of CMF was that there are two size classes of CMF. Both size classes can independently induce the expression of prespore and prestalk genes and do not need to be combined for activity. To examine their developmental regulation, we used ultrafiltration to determine the amount of the two CMFs in the conditioned medium from cells starved for different amounts of time. This experiment showed that 80 kDa CMF first appears in the buffer from cells starved for 5 hours, while small CMF can be detected only in buffer conditioned by cells for 20 hours. To characterize the small CMF, ultrafiltration and gel filtration chromatog-

raphy were used to obtain a fraction containing small CMF uncontaminated by 80 kDa CMF. We found that the small CMF was protease-sensitive. Chromatography on Concanavalin A-conjugated agarose (to which 80 kDa CMF binds) and on Sephadex G-25 showed that small CMF consists of at least 3 peptides that bind to ConA and 4 peptides that do not, suggesting that the small CMFs are a family of polypeptides[60]. We found that small CMFs could be generated by taking a >50 kDa fraction of whole CM and incubating it at room temperature for 48 hours. Small CMFs generated this way coeluted with small CMFs from whole CM as determined by sieving chromatography followed by reverse phase HLPC, suggesting that the small CMFs are breakdown products of the 80 kDa CMF[60]. Similar experiments with purified CMF indicated that the breakdown requires a high molecular weight component of conditioned medium and is not autocatalytic. Interestingly, dilution curves showed that when the 80 kDa CMF breaks down, its specific activity increases 25 to one hundredfold[60]. The data thus suggest that 80 kDa CMF is secreted by starved cells and is used for density sensing during development, and then breaks down to a higher specific activity form, perhaps to allow cells which may have been at too low a density to accumulate enough 80 kDa CMF to differentiate in some way.

CMF is produced by prespore and prestalk cells

To determine if CMF is secreted only by prestalk or prespore cells, indicating that it may be used to signal the presence of one of these cell types, we utilized the observation that cells starved in the S or early G2 phases of the cell cycle (*Dictyostelium* has no G1 phase) differentiate into prestalk and null cells while cells starved in late G2 and M become prespore and null cells[17,38,56]. Ax-4 cells were synchronized by three different methods: release from stationary phase, temperature shift, and nocodazole block. Populations of cells were harvested at 8 to 10 different points in the cell cycle. The cells were starved in shaking culture as described above to produce conditioned medium, which was then assayed for CMF activity. We found that CMF is secreted from cells starved at all phases of the cell cycle with the exception of M phase, indicating that both prespore and prestalk cells secrete CMF[59]. This experiment could not determine the secretion of CMF by null cells, which are produced by starvation of cells at any phase of the cell cycle[17]. Immunofluorescence staining of vegetative and early developing *Dictyostelium* cells using an anti-CMF antibody shows that all cells at these stages contain CMF, indicating that null cells contain and thus should be able to secrete CMF.

CMF secretion does not require pulses of cAMP

Relayed pulses of extracellular cAMP mediate both chemotaxis and the expression of many early developmentally regulated genes in *Dictyostelium*. At the high cell densities we use to make CMF, cAMP pulses could be relayed and thus potentially regulate CMF secretion. Such a regulation would indicate that CMF mediates density sensing only after aggregation has begun. We found that there is an up to twofold increase in both CMF activity as well as the amount of purifiable 80-kDa CMF in the buffer conditioned by cells pulsed with cAMP[59]. Cells lacking the cAMP receptor 1 (cAR1) due to homologous recombination gene disruption[51] secrete CMF at the basal level but fail to increase secretion when cAMP pulses are added. Two conditions can be used to block the *Dictyostelium* cAMP relay system. First, caffeine, rather than blocking phosphodiesterase as in mammalian cells, blocks the activation of adenylyl cyclase by the cell-surface cAMP receptor[4,47]. Second, high continuous levels of extracellular cAMP prevents cycling of the cAMP receptor and thus inhibits cAMP pulsing[15,16,34,39,53]. Although caffeine blocks the increased CMF secretion caused by cAMP pulses, neither caffeine nor high continuous levels of cAMP caused a decrease in the basal amount of CMF secreted by cells[59]. The data thus indicate that cAMP pulses, acting through the cAMP receptor-activated adenylyl cyclase, potentiate but are not necessary for CMF secretion.

CMF potentiates its own secretion; cell-cell contact has little effect

When starved, *Dictyostelium* cells find themselves at a variety of initial cell densities, extracellular pHs and light levels, any of which could conceivably influence the CMF secretion rate. We found that extracellular pH or light level have no effect on CMF secretion[59]. With respect to density, all of the studies on CMF used conditioned medium made by cells at a high density (10^5 cells/cm^2). At these densities, the cells are for the most part in small aggregates, or in shaking cultures touch one another quite frequently. It has been suggested that the expression of some genes during *Dictyostelium* development requires cell-cell contact[13]. To determine whether the secretion of CMF requires cell-cell contact, we starved cells at densities ranging from three times higher than the standard density of 1×10^5 cell/cm^2, down to 1.8×10^3 cells/cm^2. At the lowest density, very few of the cells ever touch one another[17]. The medium conditioned by the starved cells was collected, concentrated by ultrafiltration and assayed for CMF activity. We found that the low density cells secreted CMF, and that the amount of CMF secreted per cell increases somewhat with cell density (approximately ninefold over a 150-fold range of cell densities). Addition of purified CMF to very low density starved cells caused the amount of CMF secreted by those cells to increase. This result suggests that the increased amount of CMF secreted by high density cells is due to autostimulation and not to cell-cell contact[59].

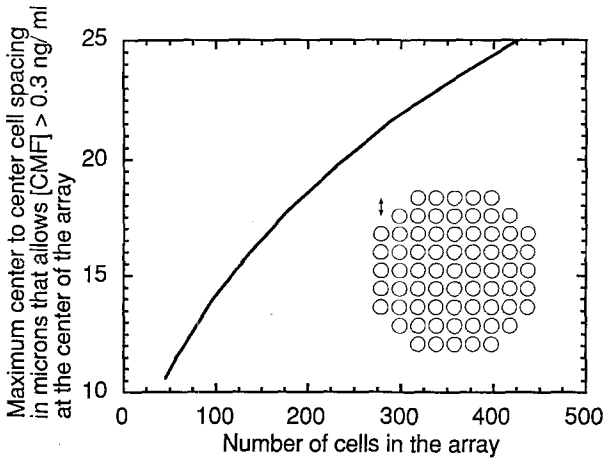

Figure 2. Diffusion calculations indicate that a diffusible substance can be used to sense or regulate the size of a tissue. In the calculations, cell secreting CMF at a constant rate of 12 molecules/cell/minute were in a flat disk with the cells in the disk in a square array; the insert shows an array of 69 cells. For the calculations, the disk of cells was on the surface of a thick moist material with the permeability properties of wet dirt. The spacing of the cells (doubleheaded arrow in inset) was varied (the cell diameter is 10 μm so the minimal center to center cell spacing is 10 μm), and the total number of cells in the array was also varied. The curve shows, for any number of cells, the largest possible center to center cell spacing for which the CMF concentration at the center of the array is at least 0.3 ng/ml, the threshold concentration for CMF activation. When the cells are touching (10 μm center to center cell spacing), a minimum of about 45 cells is needed to reach a threshold concentration of CMF. An alteration in the secretion rate, diffusion coefficient or sensitivity threshold of the CMF ligand would then alter the number of cells at which the threshold concentration is reached and permit the cells to sense whether or not that number of cells is present in the tissue.

Diffusion calculations show that CMF can be used for density sensing on a soil surface

Most of the work done showing that cells at low cell densities cannot differentiate involved cells in buffer. In the wild, cells are on a soil surface and one can envision an isolated cell continuously secreting CMF until the concentration of CMF builds up in the vicinity of the cell to the point where the cell is autostimulated and can then differentiate. To determine whether an isolated cell can secrete enough CMF to stimulate itself, we calculated the diffusion of CMF from a cell in two different geometries: sitting on a soil surface or submerged in a thin layer of water (as would be found on the surface of a leaf). A dilution curve of the purified CMF showed that it has half-maximal activity at 0.3 ng/ml[19]. Using a CMF secretion rate of 12 molecules/min, the diffusion calculations showed that the concentration of CMF in the immediate vicinity of a starved cell remains below 0.3 ng/ml by a factor of about 10 even after 10 hours of continuous secretion in either geometry, and after correcting for the presence of CMF receptors (see below) which would tend to adsorb the secreted CMF and therefore reduce the extracellular CMF concentration. Similar calculations with an array of 2046 cells showed that a CMF concentration adjacent to the cells of 0.3 ng/ml can be reached after 2 hours of secretion, again in either geometry[59]; these calculations were verified experimentally by starving cells at different densities on stacks of moist filter paper and assaying for differentiation. Interestingly, for aggregates of fewer than roughly 45 cells, the CMF concentation can never rise to 0.3 ng/ml (fig. 2). This indicates that a diffusible substance secreted and sensed by cells can form the basis for a mechanism of sensing the total number of cells in a group. We also found that the solutions are not unique: many combinations of secretion rate, diffusion coefficient, and threshold sensitivity will allow density sensing. These results showed that as a general principle cells can sense their local density by simultaneously secreting and recognizing a molecule. In addition, such a mechanism could be used for determining the total number of cells in a tissue.

CMF is required for aggregation but not for cell motility

To gain insight into the function of CMF, we made cells that lacked CMF. Fragments of the CMF cDNA were cloned into a transformation vector in an antisense orientation under the control of a *Dictyostelium* actin promoter. This construct was transformed into cells and transformants were isolated which did not secrete CMF, did not contain sequestered CMF in the vegetative cells as determined by bioassays and by Western blots, and did not contain CMF mRNA as determined by Northern blots[25,27]. When starved, the antisense transformants did not aggregate except when starved on filter pads soaked with purified CMF (starvation of untransformed cells on pads soaked with CMF appears to speed up development slightly). The above results were obtained with two different regions of the CMF cDNA in two different transformation vectors[27]. The ability of purified CMF to rescue the CMF antisense transformants and the absence of sequestered CMF in these cells shows that the gene which we have cloned encodes CMF, complementing the observation that bacterially synthesized CMF has CMF activity. These studies also confirmed that extracellular CMF concentrations above 0.3 ng/ml are required for aggregation.

The inability of CMF antisense transformant cells to form aggregates could be due to a defect in motility or a defect in the control of the motility. To determine in which of these two broad categories the CMF control is found, we first examined the motility of CMF-deficient cells. We used a timelapse video system that allows recording of cell movements at low and high magnification. A TV camera connected to a Sony Watchcorder timelapse videocassette recorder observed cells through a Nikon inverted microscope. We observed cells in two modes. In the first, to observe gross translational movements of cells, the recorder was set to record one frame

every eight seconds (when played back, events are speeded up 480-fold) and cells were observed with a 4× objective, giving a 1 mm field of view. This allows analysis of the average translational speed of the cells, which for both CMF antisense cells and untransformed cells at low density in buffer containing CMF is about 19 mm/hour (about two cell diameters/hour) immediately after starvation and then steadily decreases to about 6 mm/hour at 15 hours after starvation. These values were determined by measuring the location of cells at two-hour intervals and thus is not a measure of the path length of a cell, which because of the random movement of cells would be considerably larger (when cells are moving in a fixed direction, such as during aggregation, they can travel 1000 mm in 10 hours, or at a speed of 100 mm/hour). We also observed cells at a higher magnification and speed, using a 40× phase contrast objective and recording at four frames per second. This allowed us to observe pseudopod extension and retraction, which we find to occur about four times per minute for both CMF antisense cells and untransformed cells at low density in buffer containing CMF. We observed that during the first six hours after starvation, both CMF antisense and untransformed cells starved at low density in the absence of CMF move as fast and extend pseudopods about as often as cells starved in the presence of purified CMF. Between six and 12 hours after starvation, during which time aggregation is completed, the absence of CMF causes both untransformed and CMF antisense cells to move about half as fast and extend pseudopods about half as often as cells starved in the presence of CMF. Thus CMF, which dramatically affects aggregation, does not strongly affect the speed of cell movement.

CMF regulates cAMP signal transduction

At approximately the developmental stage (aggregation) where CMF is inducing the expression of early genes such as discoidin, pulses of cAMP are mediating both chemotaxis and the expression of a number of other early developmentally regulated genes. To understand what CMF does, we have begun to examine whether the CMF and cAMP pulse-mediated regulatory systems affect one another. As described above, we know that cAMP pulses increase CMF secretion somewhat; the observation that CMF is required for aggregation and chemotaxis suggests that CMF may in turn regulate the cAMP pulse signal transduction system. In collaboration with Dr. Peter J. M. van Haastert (University of Groningen, Holland) we have found that CMF appears to regulate several aspects of cAMP signal transduction.

Preliminary characterization of the CMF receptor

The diffusion calculations and common sense indicate that the critical parameters for a density sensing mechanism are the secretion rate of the signal, the diffusion coefficient and the sensitivity level for cells for the signal. To examine the molecular basis for the latter, and to examine how a density sensing signal is sensed, we have begun to examine the CMF receptor[25]. We have taken advantage of our observation that recombinant CMF, although more labile than purified CMF, has a molar specific activity approximately the same as that of native CMF. Following Gomer and Lazarides[18] we radiolabelled recombinant CMF with ^{125}I. For binding, we used CMF antisense cells starved for four hours, and added labelled CMF in starvation buffer. After incubating at room temperature, this was then layered on 0.3 M sucrose in starvation buffer and centrifuged. The tubes were then frozen in dry ice-ethanol and the bottoms cut off, and the radioactivity of the tube bottoms counted. After determining a crude R_T and K_D, we did a binding time course with a CMF concentration half of the K_D, and found that steady state binding is reached in 30 minutes. We chose a binding time of 30 minutes for the assays and found that the binding of ^{125}I recombinant CMF is competed for on an apparently equimolar basis by purified CMF, but is not competed off by BSA, and BSA iodinated in parallel with the CMF shows negligible binding to cells. In addiition, the subregion of CMF that competes with CMF for binding to cell surfaces is the subregion that has CMF activity in bioassays. A CMF binding saturation curve leveled off at approximately 39,000 molecules per cell, and showed half-maximal binding at a CMF concentration of approximately 170 ng/ml (2.1 nM). The extracellular CMF concentration that gives maximal activity is 1 ng/ml. At this concentration, there would be only about 200 CMF receptors occupied per cell. This suggests the existence of a cell-surface CMF receptor. For comparison, there are two interconverting forms of the cAMP receptor, one with a K_D of about 9 nM and 10,000 receptors per cell, and the other with a K_D of about 160 nM and 200,000 receptors per cell[28,52]. We have used the observed values for the number of CMF receptors per cell and their K_D in our diffusion calculations, and have found that they allow density sensing to take place (see above).

Concluding remarks

The most powerful method of defining the functions of PSF, CMF, and any other density-sensing factors yet to be identified is through mutational analysis. Isolation of the CMF cDNA allowed construction of an antisense CMF 'null' strain, whose properties revealed the essential role of CMF in aggregation. Isolation of the PSF gene should be similarly informative. Additionally, it is not known whether PSF-mediated gene expression is essential for development, and this issue too is unlikely to be resolved until mutants lacking PSF become available. Related questions are how responsiveness to PSF

is controlled, and whether the most and least responsive cells in a population differ in developmental fate. Meanwhile, even our limited understanding of these autocrine signaling pathways has revealed that *Dictyostelium* amoebae sharing the same local environment communicate with each other and modulate their behavior in concert. In this sense, even during growth, *Dictyostelium* never entirely loses its properties as a multicellular organism.

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