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Source: Zoological Science, 12(1) : 61-69

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.12.61

ZOOLOGICAL SCIENCE 12: 61–69 (1995)

A Proteinous Factor Mediating Intercellular Communication during the Transition of *Dictyostelium* Cells from Growth to Differentiation

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ABSTRACT—In general, cell differentiation and proliferation are mutually exclusive. Transition of the cellular slime mold *Dictyostelium discoideum* from growth to differentiation is triggered mainly by a secreted factor(s) in addition to nutritional deprivation. To purify and identify the factor required for the growth/differentiation transition, a new assay system was designed. Under low-nutrient conditions, cells could grow to multiply, but never developed. The cellular development including aggregation, however, was induced by the addition of conditioned medium (CM) in which growing or starving *Dictyostelium* cells had been cultured. The CM inhibited the synthesis of nuclear DNA and induced the cells to acquire chemotactic competence to cAMP, thus suggesting the presence of a secreted factor(s) required for growth/differentiation transition in the CM. The active factor(s) in CM (referred to as CMF^{450}; conditioned medium factor) was found to be sensitive to heat and have a large molecular size. The CMF^{450} was purified using FPLC through a gel filtration column, and was identified to be a proteinous macromolecule of *Mr* 450 kDa, which was mainly composed of 94 kDa, 79 kDa, and 49 kDa subunits under a native condition.

INTRODUCTION

In general, cellular differentiation goes against cell proliferation, and the transition from one to the other is a critical issue to be solved in cell biology and developmental biology. The growth/differentiation transition is sometimes mediated by intercellular communications through diffusible substances secreted by cells. The cellular slime mold *Dictyostelium discoideum* grows and multiplies as free-living amoebae as long as nutrients are available. Upon nutritional deprivation, starving cell populations initiate a developmental program including cell aggregation. During aggregation, cells acquire aggregation competence and aggregate to form multicellular structures by chemotaxis to 3′,5′-cyclic adenosine monophosphate (cAMP)[reviewed in 8]. The cell aggregate exerts a series of morphogenesis to form a migrating slug that eventually develops to a sorocarp consisting of a mass of spores and a supporting cellular stalk. Thus the growth and differentiation phases are temporarily separated from each other and easily controlled by nutritional conditions. This is a great merit to analyze the mechanisms of growth/differentiation transition at the cellular and molecular levels.

With respect to the growth/differentiation transition, it has been claimed using synchronized *D. discoideum* AX-2 cells that cells progress through the cell cycle to a particular point in mid-late G2 phase (referred to as a putative shift point; PS-point), irrespective of the presence or absence of nutrients, and enter the differentiation phase from this point in response to nutritional deprivation [17]. Particularly around the PS-point, the significance of phosphorylation states of several proteins [2, 7] and also that of specific gene expression [1] have been argued.

Nutritional deprivation is essential for the initiation of cellular development, but not enough. In the *D. discoideum* wild-type strain NC-4, several genes including *discoidin-I* are actually induced not by starvation *per se*, but by a mechanism that measures cell density relative to nutrient supply [5]. As shown by Clarke and coworkers [6], *Dictyostelium* cells continuously secrete a factor (PSF for "prestarvation factor") during the growth phase and accumulate it as a function of cell density. PSF, a proteinous substance of *Mr* 65–70 kDa, induces the synthesis of certain proteins that were previously believed to be induced by starvation: the most prominent is discoidin-I [5]. We recently found that exponentially growing AX-2 cells synthesize and release a proteinous macromolecule(s) of *Mr* 30–40 kDa (referred to as PSF' for convenience) that accumulates in a cell-density-dependent manner and enables cells to develop after starvation [18]. Because of difficulty in purifying biochemically PSF and PSF', their chemical structures remain to be elucidated.

During the first few hours of starvation, *Dictyostelium* cells secrete a macromolecule (presumably a glycoprotein of *Mr* 70 kDa) that stimulates the ability of low-density starving cells to differentiate and thus is named DSF (differentiation-stimulating factor) [11]. Another secreted factor, which is called CMF (conditioned medium factor), is detectable in conditioned medium within 0.5 hr after the onset of starvation [21]. In very low-density monolayer culture of starving AX-2 cells, the CMF and cAMP are both required for cell-type-specific gene expression in later development [21]. Gomer *et al.* [9] and Yuen *et al.* [26] have shown by size-fractionation of CMF that the activity is separated into high and low *Mr* fractions, and that the large CMF is a single

Accepted December 5, 1994
Received September 26, 1994

80 kDa protein. In this study, we report a new macromolecule of 450 kDa (referred to as CMF^{450}), which is secreted by starving AX-2 and NC-4 cells and responsible for the initiation of cellular development. The relationship between the CMF^{450} and other factors such as CMF and DSF is discussed, with reference to their chemical nature and physiological functions.

MATERIALS AND METHODS

Organisms and cultivation

Dictyostelium discoideum axenic strain AX-2 (clone 8A) grown in HL-5 medium supplemented with 1.5% glucose was mainly used in this study. *D. discoideum* wild-type strain NC-4 was also used for the preparation of conditioned medium. NC-4 cells were grown with *Escherichia coli* on SM agar (glucose 1%, Bacto-peptone (Difco) 1%, yeast extract (Difco) 1%, $MgSO_4 \cdot 7H_2O$ 0.1%, KH_2PO_4 0.19%, K_2HPO_4 0.1%, and Bacto-agar (Difco) 2%). For the assay of PSF activity, NC-4 cells as test cells were suspension-cultured with *Klebsiella aerogenes* suspension at 150 rpm at 22°C.

Preparation of conditioned medium

Conditioned medium (CM) was prepared, basically according to the method of Gomer *et al.* [9]. Stationary-phase AX-2 cells were harvested at 1×10^7 cells/ml and collected by centrifugation for 2.5 min at 340 g. The cell pellet was suspended, washed twice in PBM (20 mM Na/K-phosphate, 0.01 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 6.2), and then resuspended in PBM at a density of 1×10^7 cells/ml. After 16 hr of shake culture at 22°C, the cell suspension was centrifuged at 37000 g for 30 min and the resulting supernatant was stored as conditioned medium (CM) with 1.5 mM sodium azide at 4°C. In another experiment, NC-4 cells grown with bacteria were harvested, collected and washed free of bacteria by four times of differential centrifugations in PBM. The CM was prepared as described above.

The assay of conditioned medium activity

Exponentially growing AX-2 cells ($2–4\times10^6$ cells/ml) were collected by centrifugation for 1 min at 940 g, and washed once in BSS (Bonner's standard salt solution) [4]. The washed cells were suspended in BSS containing a 1/20 concentration of HL-5 medium at 2.7×10^5 cells/ml. An aliquot (150 μl) of the cell suspension was mixed with 50 μl of CM (positive control), PBM (negative control), or fractionated sample to be tested for the CM-activity in a 96-well plate (Corning). After 12 hr of incubation at 22°C, the development of cells was monitored under a phase-contrast microscope. When aggregation-streams or cell masses at more advanced developmental stages were formed, the activity of CM was scored as positive. The strength (unit) of the CM was represented as an inverse number of the highest dilution at which the activity was counted as positive.

Gel electrophoresis and staining

Samples (10–15 μl) were loaded on SDS-polyacrylamide (7.5% slub gel of 1 mm thickness) and electrophoresed according to Laemmli [12]. In another experiment, native PAGE was carried out. After electrophoresis, proteins in the gels were visualized by silver staining according to a slight modification of Poehling's method [22]: The gels were fixed in 6% glutaraldehyde for 40 min at 32°C before silver staining. To purify CMF^{450} (conditioned medium factor) as an active form, the sample was loaded on a disc gel (5 mm diameter, 6 cm hight) composed of 4% acrylamide as a stacking gel and 7.5% acrylamide as a separation gel. After electrophoresis, the gel was cut into 2 mm slices, and crashed by passing through a nylon mesh. This was followed by extraction of proteins by soaking each crashed gel overnight in PBM.

Fractionation of proteins

Almost all of the following processes were done at 4°C. The original CM was concentrated using a 100×10^3 *Mr* cutoff polysulfone membrane (Millipore Ultrafiltration System). The concentrated CM was dialyzed overnight against 20 mM Na/K phosphate buffer, pH 6.4 (PB), with several exchanges of PB. The CM preparation was applied onto 2×15 cm DE-32 column (Whatman) equilibrated with PB and then eluted from the column with 250 ml-NaCl gradient (0–400 mM). Fractions containing strong CM activity were loaded on 1.2×15 cm Phenyl-Sepharose CL-4B (Pharmacia) equilibrated with 1.5 M ammonium sulfate in PB. This was followed by elution of bound materials with a gradient (1.5–0 M) of ammonium sulfate in 80 ml PB. Mono Q, Superdex 200 and Superose 6 columns (Pharmacia) were run on a Pharmacia FPLC system at room temperature, equilibrating with PB. The elution from Mono Q was done with a NaCl-gradient (0–500 mM).

Determination of the N-terminal amino acid sequence of isolated proteins

The active CM fractions eluted from the Phenyl-Sepharose column were evaporated by a centrifugal concentrator and dissolved in 5 μl of 1×SDS-PAGE loading buffer (10 mM Tris-HCl pH 6.8, 10 mM dithiothreitol, 10% glycerol). The sample was loaded onto SDS-PAGE (7.5%) and electrophoresed, followed by electroblotting to PVDF membranes (PALL). After staining of the membrane with Coomassie Brilliant Blue (CBB), the membrane was dried, and the bands with CMF^{450} were withdrawn. This was followed by determination of the N-terminal amino acid sequence of CMF^{450} using a protein sequencer (Applied Biosystems, 477A).

Chemotactic assay

The chemotactic sensitivity of cells to cAMP was assayed by a slight modification of the method of Maeda and Maeda [16]. CMF^{450}-treated and non-treated cells were separately washed once by centrifugation in BSS, and droplets (10 μl) of the cell suspensions were placed on agar (1.5% Difco, Purified) containing BSS. Subsequently, agar blocks containing various concentrations (1×10^{-5}, 1×10^{-6}, and 1×10^{-7} M) of cAMP were placed near the cell droplets. After 30–60 min of incubation at 22°C, the response was scored as positive if the periphery of droplets neighboring the cAMP-agar blocks had thickened, resulting from chemotactic movement of cells.

DNA-specific staining with DAPI

Cells with and without CMF^{450}-treatment were separately withdrawn and washed once by centrifugation in BSS. For fixation of cells, ice-cold absolute methanol was added into the cell suspensions and centrifuged. The resulting cell pellets were resuspended in ice-cold absolute methanol and refixed in an ice-bath for 10 min [3]. One drop of the cell suspension was put on a cleaned coverslip and dried by hot air using a dryer. The fixed cells were stained with DAPI as described previously [13, 15]. The percentages of mononucleate cells were determined using UV excitation under a fluorescence microscope. At least 1000 cells were counted for each.

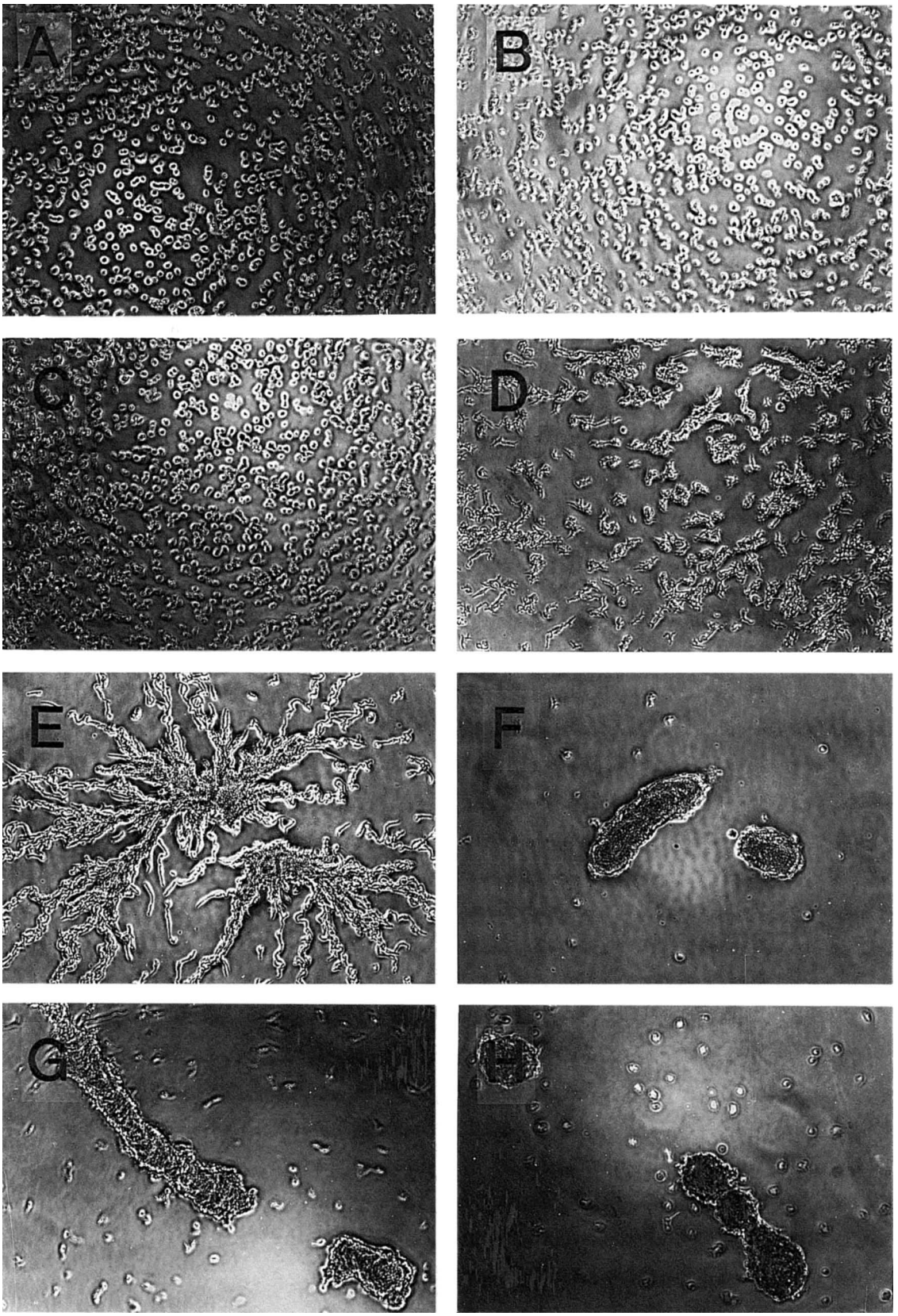

FIG. 1. Inhibitory effect of nutrients on the progress of development. *D. discoideum* AX-2 (clone 8A) cells at the growth phase were harvested, washed once in BSS and settled on a 96-well plate at 1.2×10^5 cells/cm^2. Subsequently, various amounts of nutrient medium (HL-5) were added. A, B, a 1/20 concentration (1/20 HL-5) of HL-5; C, D, 1/320 HL-5; E, F, 1/1280 HL-5; G, H, no HL-5. Cells were incubated for 10 hr (A, C, E, G) or 18 hr (B, D, E, H) at 22°C. In the presence of 1/20 HL-5, cells showed no sign of development. Observations under a phase-contrast microscope. ×71.

Assay of PSF activity

NC-4 cells at the growth phase were harvested, washed once in BSS, and then transferred to freshly prepared *K. aerogenes* suspension with or without CMF^{450}. After about 12 hr of shake culture at 150 rpm at 22°C, NC-4 cells were withdrawn and washed by centrifugation in BSS for the subsequent PSF assay. The activity of PSF is usually monitored by its stimulation of discoidin-I synthesis in growing NC-4 cells [5]. The amount of intracellular discoidin-I was visualized using an indirect immunocytochemical method. For this, washed cells were fixed in absolute methanol as the case for DAPI-staining in the preceding section. The fixed cells were reacted with two kinds of monoclonal antibodies (5C7, 3G4) raised against discoidin-I, which were generously gifted from Dr. M. Fukuzawa of Hokkaido Univ., overnight at 4°C. After three times of washings in PBS (10 mM Na/K phosphate buffer containing 0.9% NaCl, pH 7.0) for 7 min for each, the preparations were stained with a fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG goat antibody (TAGO, Inc.) (60×diluted) overnight at 4°C, followed by extensive washings in PBS. They were mounted on a slide glass with a small amount of PBS containing 10% glycerol. The fluorescence intensities in CMF^{450}-treated cells and non-treated cells were observed and compared under a fluorescence microscope using B2 excitation.

RESULTS

CM activity required for the initiation of development

Dictyostelium cells start their development in response to complete nutritional deprivation. When some amino acids are added to starvation medium like PB, cellular development is known to be greatly delayed [20]. As was expected, the addition of nutrient medium (HL-5) to PB was found to delay the development of AX-2 cells depending on the amount of nutrients (Fig. 1). At a certain cell density (1.2×10^5 cells/cm^2), a 1/20 concentration (1/20 HL-5) of HL-5 inhibited completely the cellular development including cell aggregation. When the cell density was doubled, however, AX-2 cells progressed their development even in the presence of 1/20 HL-5 (Fig. 2). This seems to indicate that maybe a diffusible substance(s) secreted by cells are able to overcome the nutrient-containing condition that must be unfavorable for the initiation of development. In fact, the addition of original CM (derived from NC-4 or AX-2 cell suspensions) to 1/20 HL-5 allowed cells to develop basically depending on the CM-concentrations (Fig. 3). The application of highly concentrated CM, however, had rather an inhibitory effect on cell aggregation. Although the reason for this inhibition is presently unknown, it is possible that highly concentrated CM might be utilized as nutrients, thus resulting in inhibition of cellular development.

The nature of CM activity was preliminarily examined. The CM activity was completely lost within 1 min by heat-treatment at 100°C. At a lower temperature around 80°C, the activity was lost within 10 min, though most of the activity was retained at 37°C for at least 3 hr. Thus, it is most likely that the active CM component may be a proteinous macromolecule(s).

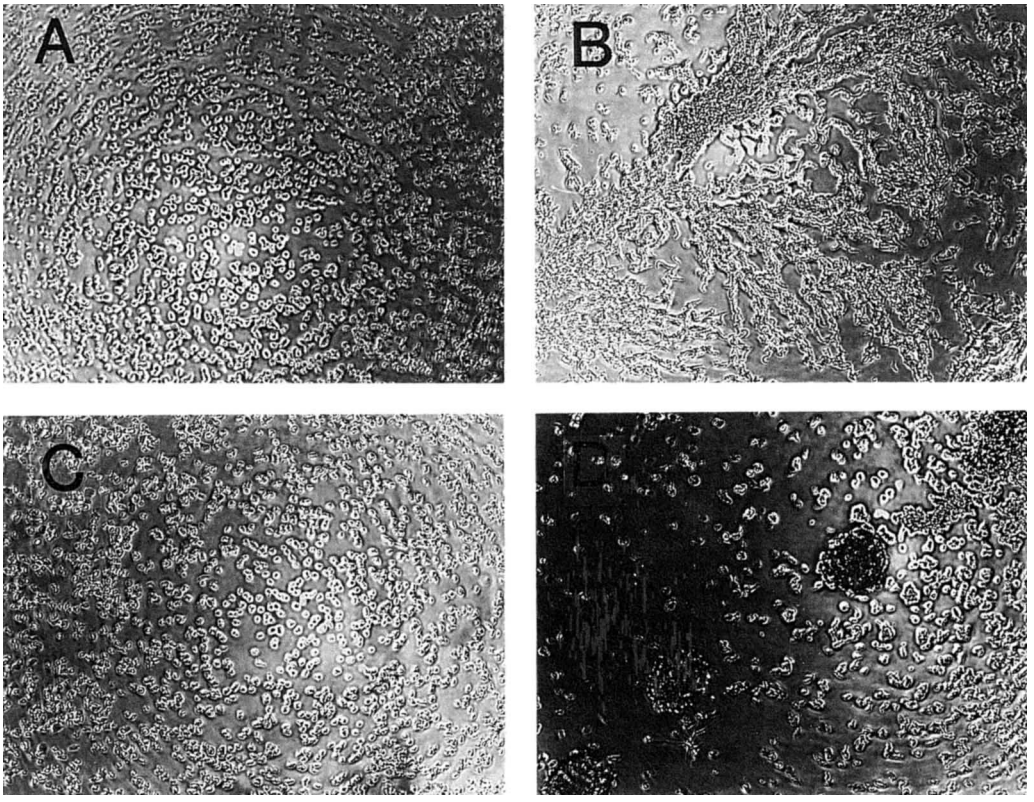

FIG. 2. Dependency of the developmental progress on cell densities in low-nutrient cultures. At a higher cell density (2.4×10^5 cells/cm^2) (B, D), AX-2 (clone 8A) cells were able to develop even in the presence of 1/20 HL-5, thus being in contrast with those at a lower cell density (1.2×10^5 cells/cm^2) (A, C). Cells were incubated for 18 hr (A, B) or 30 hr (C, D) at 22°C. ×88.

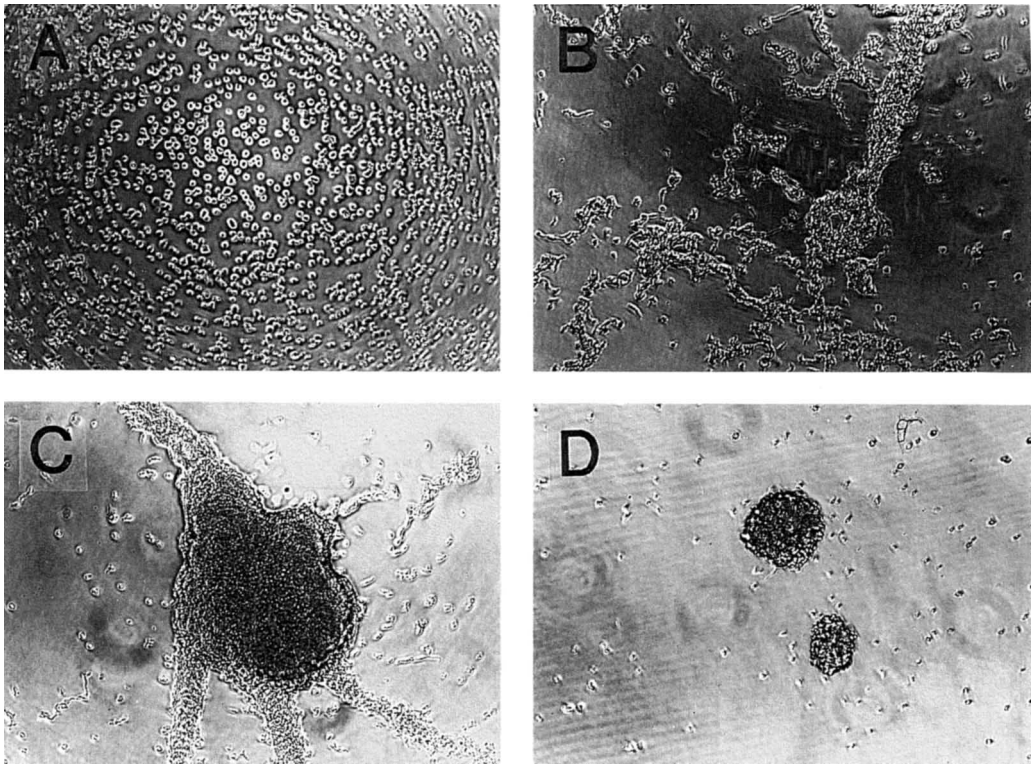

FIG. 3. Effect of conditioned medium (CM) on the developmental progress in low- nutrient cultures. AX-2 cells were prepared as described in the legend to Fig. 1. In the presence of 1/20 HL-5, various concentrations of CM (A, no CM; B, 5 units; C, 20 units; D, 80 units) were applied and incubated for 18 hr at 22°C. It is evident that cell aggregation is induced depending on the CM-concentrations. ×50.

Purification and chemical nature of CMF^{450}

To purify the active CM component (CMF^{450}), CM prepared from NC-4 cell suspensions was concentrated and dialyzed, followed by loading on an anion exchange column DE-32. The CM-activity assay of fractions eluted from the column showed that most of the CM activity is present between fraction No. 41 and 46 (Fig. 4A). The fractions (41–46) were then chromatographed on a Phenyl-Sepharose column and assayed after dialysis. Almost all of the activity was eluted between fraction No. 38 and 46 (Fig. 4B). Subsequently, highly active fractions (38–42) were concentrated by the use of Microcon (cutoff 10 kDa, Millipore) and applied to FPLC using a gel filtration column, Superdex 200 or Superose 6 (Fig. 4C). The highest activity was detected in the fraction of *Mr* 450 kDa. When this component was further loaded on Mono Q, a single peak was obtained (Fig. 4D). The protein content and specific CM activity at each step of the purification procedure were summarized in Table 1.

The fraction with the highest CM activity was then applied to native-PAGE and SDS-PAGE for further purification of CMF^{450}. After electrophoresis, one major band was noticed in native-PAGE, but three major bands in SDS-PAGE (Fig. 5). Apparent molecular weights of the three bands in SDS-PAGE were estimated to be 94, 79, and 49 kDa. Essentially the same results were obtained using CM prepared from AX-2 cell suspensions. When CM derived from AX-2 cells was used as a starting material, an active fraction eluted from Mono Q was found to be contain many minor bands besides the major three bands in SDS-PAGE. Therefore, the CM from AX-2 cells might be cruder than that from NC-4 cells. In a preliminary experiment, growing NC-4 cells also were found to secrete the CMF^{450}, though the amount of CMF^{450} secreted was considerably less as compared with that from starving NC-4 cells. Taken together these results suggest that the CMF^{450} may be a large protein complex consisting of at least three subunits (94 kDa, 79 kDa, and 49 kDa proteins), though the precise chemical structure of 450 kDa CMF^{450} is presently unknown. When the three proteins eluted from the SDS-gel were separately assayed for CM activity to test which subunit is active, none of them showed CM activity. Therefore either a big complex of *Mr* 450 kDa or a minor component(s) other than the three proteins is most likely to be responsible for CM activity. To determine the N-terminal amino acid sequence of the subunit structures of CMF^{450}, 94 kDa, 79 kDa, and 49 kDa proteins separated by SDS-PAGE were blotted onto a PVDF membrane, followed by staining of the membrane with CBB. The stained membrane strips corresponding to the three proteins were separately analyzed for the N-terminal structures. As a result, the N-termini of 94 kDa and 79 kDa proteins were found to be chemically modified and the sequences failed to be determined. On the other hand, the N-terminal sequence of 49 kDa protein was determined as EQNEDKDDDFSGTH.

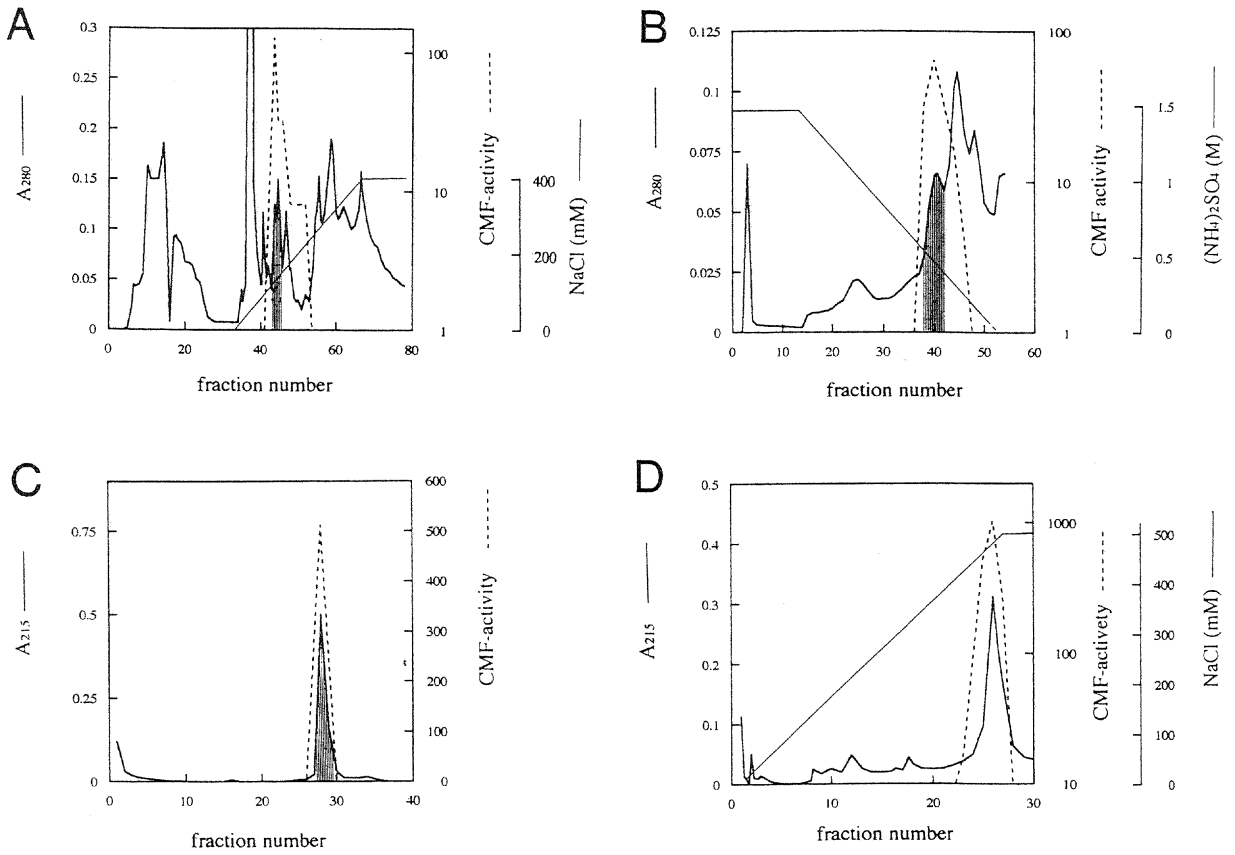

FIG. 4. Elution patterns of CMF^{450} from various fractionation columns. Concentrated and dialyzed CM derived from NC-4 cell suspensions was applied to DE 32 (Whatman), and the resulting bound materials were eluted by 0–400 mM NaCl gradient (A). Eluted fractions with relatively high CM activity (shadowed area) were then applied to Phenyl-Sepharose CL-4B column and eluted by 1.5–0 M ammonium sulfate (B). The fraction with the highest CM activity (shadowed area) was applied to Superose 6 (C). Notice a single peak with strong CM activity. The fraction corresponding to this peak was finally applied to Mono Q. A 0–500 mM NaCl gradient was used for elution (D). The solid line, absorbance at 280 nm (A280) in A and B, absorbance at 215 nm (A215) in C and D; the broken line, CM activity.

TABLE 1. Purification of an active conditioned medium factor (CMF^{450}) Data from Fig. 4

Fractionation	Protein content (mg)	Specific activity of CM (units/mg protein)
Initial material (concentrated CM)	5144	0.3×10^3
DE32	21.1	1.8×10^3
Phenyl-Sepharose	3.45	2.2×10^3
Superose 6	0.11	9.8×10^4
Mono Q	0.10	2.3×10^5

Function of CMF^{450} at the cellolar level

To examine effect of CMF^{450} on the acquisition of chemotactic ability to cAMP, chemotactic sensitivities of AX-2 cells incubated with or without CMF^{450} were compared. In the absence of CMF^{450}, cells showed no sign of chemotaxis to cAMP. CMF^{450}-treated cells, however, exhibited positive chemotactic movement toward agar blocks containing 10^{-5}–10^{-7} M cAMP (data not shown). About 20% of AX-2 cells growing in axenic cultures were multinucleate. Staining with DAPI revealed that almost all the nuclei contained twice the amount of DNA present in daughter nuclei of anaphase mitotic figures as described previously [17], thus indicating little or no Gl-phase in the cell cycle (data not shown).

As shown in Fig. 6A, the cell number increased, irrespective of the presence or absence of CM, with almost the same kinetics. On microscopic observation of the cells incubated in 1/20 HL-5 containing CM, it was found that there were increases in the ratio of mononucleate cells particularly after 10 hr of incubation (Fig. 6B). Without CM, however, the ratio of mononucleate cells was rather decreased. Thus the observed increase of the CM-treated cells might be at least partly due to the conversion of multinucleate cells into mononucleate cells. In other words,

TABLE 2. Summary of secreted factors involved in intercellular communications during and just after growth in *Dictyostelium discoideum* cells

Factor	Working period	Heart stability	Relative molecular size	References
DGF	during growth	stable	retained in dialysis bag >1.2 kDa	Whitbread *et al.* [25]
PSF	during growth	unstable	similar to BSA 65–70 kDa	Clarke *et al.* [5]
PSF'	during growth	stable	30–40 kDa	Maeda and Iijima [18]
DSF	after starvation	stable	about 75 kDa	Klein and Darmon [11]
CMF	after starvation	stable	80 kDa+1–5 kDa	Gomer *et al. [9]*
CMF^{450}	*after starvation*	*unstable*	*450 kDa*	*this paper*

CMF, conditioned medium factor. DGF, *Dictyostelium* growth factor. DSF, differentiation stumulating factor. PSF, prestarvation factor.

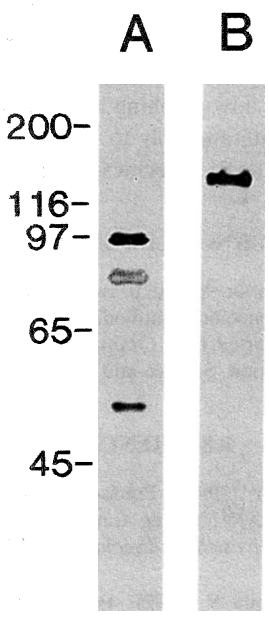

FIG. 5. 1D-gel analysis of purified CMF^{450}. One hundred units of CMF^{450} eluted from Mono Q were applied to 7.5% SDS-PAGE(A) and 7.5% native PAGE(B). After electrophoresis, gels were stained with silver.

the CM seems to enhance cytokinesis, but inhibits the synthesis of nuclear DNA.

Does CMF^{450} have PSF activity?

It is of interest to know if the CMF^{450} has PSF activity that is believed to work at the growth phase. To test this, effect of the CMF^{450} on the synthesis of discoidin-I was monitored immunocytochemically using anti-discoidin I monoclonal antibodies. The result showed that the application of CMF^{450} (10–1000 units) to exponentially growing NC-4 or AX-2 cells has no effects on the amount and localization of discoidin-I of the cells (data not shown), thus suggesting that the CMF^{450} is quite different from the PSF.

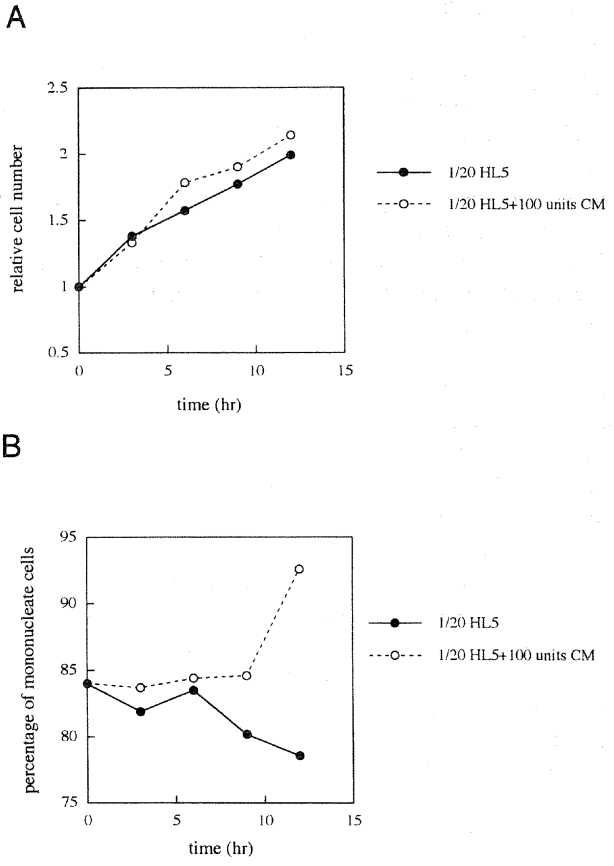

FIG. 6. Effects of CMF^{450} on cell proliferation and multinuclearity. AX-2 cells were incubated for the indicated periods in 1/20 HL-5 with or without 100 units of CMF^{450} eluted from Phenyl-Sepharose column, at a low density (1.2×10^5 cells/cm^2). (A). Changes in cell number. (B). Changes in multinuclearity monitored by DAPI-staining of cells.

DISCUSSION

Dictyostelium cells have recourse to a variety of communication systems before the establishment of cell masses, which eventually regulate growth and differentiation in a cell-density dependent manner. In cell aggregation, for

example, cAMP plays a central role as a signalling substance of chemotaxis [14]. Besides, several factors secreted by growing and/or starving cells, as summarized in Table 2, are working as diffusible intercellular communicators. In this report, we have shown that *D. discoideum* NC-4 and AX-2 cells produce and secrete a novel macromolecule (CMF^{450}) of *Mr* 450 kDa that accumulates in starvation or growth medium in proportion to cell density and is required for the transition of AX-2 cells from growth to differentiation. The CMF^{450} has protein-like characters such as heat-instability and seems to be a large protein complex consisting of at least three subunits (94 kDa, 79 kDa, and 49 kDa proteins). The CM activity was not lost by SDS-treatment before electrophoresis, but the separation of CMF^{450} into the subunit structures resulted in complete loss of the CM activity (differentiation-inducing ability). Therefore, it seems likely that whole structure of CMF^{450} may be essential for manifestation of the activity. To obtain a higher amount of CMF^{450}, it was purifed from the CM in which starved cells had been cultured for 16 hr. Since the effect of CMF^{450} was monitored at the initial step of cellular development, it is likely that the CMF^{450} is involved in the phase-shift from growth to differentiation. The CMF^{450} seemed to be continuously secreted for a relatively long period after starvation, and therefore the CMF^{450} also might play a role in later development of cells.

CMF was originally reported as a secreted factor required for cell-type specific gene expression in low-density monolayer culture of *D. discoideum* cells [21]. Recently, the CMF was purified and identified to be a 80 kDa glycoprotein (CMF-H), and its degradation products (CMF-L) of much smaller sizes were also found to have the CM activity [9, 26]. Mann and Firtel [19] have demonstrated that the CMF induces cAMP receptor 1 and a cell-adhesion molecule gp80, both of which play important roles in the establishment of multicellular structures. This was confirmed by the fact that the transformed cells expressing antisense RNA of the CMF fail to aggregate [10]. Although the CMF^{450} reported here is somewhat similar to the above CMF in that both of them are secreted predominantly after the start of development, there are marked differences in heat-stability and molecular size between the two. The synthesis of discoidin-I is induced by the CMF as well as by the PSF [9]. As presented in this work, however, the CMF^{450} never induced the discoidin-I synthesis. Thus, the CMF^{450} is concluded to differ in structure and function from the CMF.

Klein and Darmon [11] have shown the presence of a factor (DSF; differentiation-stimulating factor) that is a protein of about 75 kDa and promotes cell aggregation in low-density culture. The DSF is secreted by starving cells and sensitive to heat, but seems to be different in molecular size from the CMF^{450}.

During the vegetative growth phase, *Dictyostelium* cells secrete continuously a prestarvation factor (referred to as PSF) into growth medium and accumulate it as a function of cell density [6, 23]. The PSF is known to induce expression of genes such as *discoidin I, cAMP receptor 1* (*CAR1*), and *phosphodiesterase* during the growth phase, most of which are usually expressed after starvation [24]. We recently reported another type of PSF (PSF') that is required for the acquisition of development competence in AX-2 cells and is active with growth-phase cells only [18]. Therefore, the PSF and PSF' are genuine prestarvation factors effective only on growing cells.

As imagined from the above account, several diffusible factors work as intercellular communicators that enable starving *Dictyostelium* cells to develop normally, particularly around the phase-shift point (like the PS-point) from growth to differentiation. To understand the molecular mechanisms of growth/differentiation transition, we will need to know (1) the molecular basis of "starvation" and also (2) the precise action mechanisms of the secreted factors separately and then totally. Since the N-terminal amino acid sequence of the 49 kDa protein (one of subunits of CMF^{450}) was determined, we are now planning to isolate a gene encoding the protein and simultaneously to prepare monoclonal antibodies that can neutralize specifically the CM activity.

ACKNOWLEDGMENTS

We are grateful to Dr. M. Fukuzawa of Hokkaido Univ. for his generous supply of monoclonal antibodies against discoidin-I. This work was partly supported by a Grant-in-Aid (no. 04454018) from the Ministry of Education, Science and Culture of Japan.

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