

A novel prespore-cell-inducing factor in *Dictyostelium discoideum* induces cell division of prespore cells

Akiko A. Oohata^{1,*}, Manabu Nakagawa², Masao Tasaka^{3,†} and Shigeru Fujii²

¹Biological Laboratory and ²Chemical Laboratory, Kansai Medical University, Hirakata, Osaka 573, Japan

³National Institute for Basic Biology, Myodaiji, Okazaki 444, Japan

*Address for correspondence (e-mail: oohata@makino.kmu.ac.jp)

†Present address: Department of Botany, Division of Biological Science, Graduate School of Science, Kyoto University, Kyoto 606-1, Japan

SUMMARY

In *Dictyostelium discoideum* strain V12M2, at a very low cell density ($\sim 10^2$ cells/cm²), most amoebae differentiate into prespore cells in a salt solution containing cAMP if an adequately diluted conditioned medium (CM) is provided (Oohata, A. A. (1995) *Differentiation* 59, 283-288). This finding suggests the presence of factor(s) released into the medium that are involved in inducing prespore cell differentiation. In the present study, we report the presence of two types of factors that function synergistically in prespore cell induction; one is a heat-stable and dialysable factor(s) and the other is a heat-labile and non-dialysable factor termed psi (Ψ) factor (prespore-inducing factor). We purified and characterized

the psi factor. Its relative molecular mass was determined to be $106 \times 10^3 M_r$ by SDS-PAGE and $180 \times 10^3 M_r$ by gel filtration HPLC, respectively. These results indicate that psi factor exists as a dimer under native conditions. In addition to inducing prespore cell differentiation, psi factor induced cell division of prespore cells in submerged culture. Our results suggest that psi factor plays important roles not only in prespore cell differentiation but also in the progress of the cell cycle in the prespore pathway in normal development.

Key words: *Dictyostelium discoideum*, morphogen, differentiation, conditioned medium, cell cycle, cell division

INTRODUCTION

The cellular slime mould *Dictyostelium discoideum*, has been used as a good model system for morphogenesis and cell differentiation because of its simple development. Amoebae of this organism multiply by binary fission, feeding on bacteria, and then aggregate to form multicellular masses when the food source is consumed. The amoebae differentiate into prespore and prestalk cells, which differentiate into spore and stalk cells, respectively, in the subsequent fruiting bodies.

In *Dictyostelium discoideum*, several factors involved in differentiation have been identified and some of them have been purified and characterized (see, Williams, 1988; Oohata, 1995). The first to be identified was cAMP, which controls many essential processes of development by mediating signal transductions (for review see, Gross, 1994; Parent and Devreotes, 1996). In addition, a density-sensing factor, CMF (conditioned medium factor) and differentiation-inducing factor-1, DIF-1 (Gomer et al., 1991; Berks et al., 1991) have been well characterized.

CMF, a glycoprotein of $80 \times 10^3 M_r$, and its breakdown products are considered to function as part of a cell-density-sensing mechanism for cells of strain NC-4 and axenic strains in their normal environment (Gomer et al., 1991). At high cell densities, the extracellular concentration of CMF is sufficient to enable the cells to enter the multicellular stage of the developmental cycle. When present below a threshold concentration,

the cells do not initiate the expression of genes required for early development. However, amoebae of strain V12M2 differentiate into prespore and stalk cells in a salt solution containing cAMP even when the cell density is below that at which cell contact is recognised (Oohata, 1992). Thus, V12M2 is a convenient strain to investigate prespore and prestalk cell differentiation, excluding high-cell-density-dependent processes in early development. Using this strain, DIF-1 was discovered and purified from conditioned medium (Town et al., 1976; Kay et al., 1989). DIF-1 is a chlorinated hexaphenone and functions by entering the cells; high concentrations of DIF-1 induce prestalk and stalk cell differentiation and repress prespore cell differentiation (Kopachik et al., 1985; Early and Williams, 1988; Kay et al., 1989), while low concentrations promote prespore cell differentiation (Oohata, 1995). Therefore, DIF-1 is considered to play a key role in the differentiation of this organism.

Previous studies have also indicated the existence of dialysable and heat-stable factors that are involved in prespore cell differentiation (Weeks, 1984; Kumagai and Okamoto, 1986; Gibson and Hames, 1988). These findings suggest that DIF-1 and other factors that induce prespore cell differentiation may act mutually to control the prestalk-prespore pattern formation. However, decisive evidence is lacking.

In a previous paper, we reported the presence of a prespore-inducing substance(s) in a conditioned medium (CM) (Oohata, 1995). In this study, we further analyze the CM and clearly

show that it contains two components that are essential for prespore cell differentiation. One of them is a dialysable factor(s) and the other is a high molecular-weight protein, which not only induces prespore cell differentiation but also the division of prespore cells. The latter is a novel factor, resembling animal growth factors, which are both morphogens and mitogens.

MATERIALS AND METHODS

Cell culture

Amoebae of *Dictyostelium discoideum* strain V12M2 were grown with *Klebsiella aerogenes* on SM agar (Oohata, 1995) at 23.5°C for 28 hours.

Preparation of conditioned medium (CM)

After washing five times in Bonner's salt solution (Bonner, 1947) containing antibiotics (BSS: 10 mM KCl, 10 mM NaCl, 3 mM CaCl₂, 220 µg/ml streptomycin sulphate, 15 µg/ml tetracycline), the amoebae were incubated in 15 cm tissue culture dishes containing BSS at a density of 1.4×10⁶ cells/cm² (8×10⁶ cells/ml) at 22–23°C for 48 hours. After the incubation medium was collected, centrifuged at 2800 g for 20 minutes and filtered with syringe filter (pore size, 0.45 µm), the filtrate was stored at 4°C until used.

Bioassay of prespore-cell-inducing activity

After washing in BSS, amoebae were plated in 3.5 cm Nunc tissue culture dishes containing buffered prespore salt solution (BPSS: 10 mM KCl, 10 mM NaCl, 3 mM CaCl₂, 1 mM MgCl₂, 5 mM KH₂PO₄/K₂HPO₄ (pH 6.6), 2.5 mM Na-cAMP, 220 µg/ml streptomycin sulphate, 15 µg/ml tetracycline) at a density of 5×10² cells/cm² (5×10³ cells/ml) unless stated otherwise and incubated at 21–23°C for 22–24 hours. The cells were fixed with methanol in situ and stained with FITC-conjugated anti-*D. mucoroides* spore serum as described previously (Oohata, 1995). Prespore cell ratios were determined using phase-contrast epifluorescence microscopy as follows. Cells that contained FITC-stained granules (PSVs) in their cytoplasm were considered to be prespore cells. At least, 500 cells were scored per assay. The unit of activity of the ψ factor was defined as that which induces 1% prespore cell differentiation in the above-mentioned standard 1 ml assay in the presence of 50 µl dialysate of CM. The dialysate was a filtrate of M_r 5×10³ cutoff Ultrafree CL (Millipore, MA, USA).

Measurement of cell division

The cells were incubated in a tissue culture dish containing 1 ml of BPSS at a density of 10³ cells/cm² in the absence or presence of 500 units of ψ factor and 50 µl dialysate of CM at 25°C. Cell divisions were observed by time-lapse videomicroscopy using a Nikon TMS inverted microscope equipped with a tungsten lamp. The videotape system consisted of a Frovel high gain color camera HCC-574, a Kogaku time-lapse illuminator, a Victor time-lapse videocassette recorder SR-1900 and a Sony RGB color video monitor PVM-1440. The cells were videotaped at 10 fields/20 seconds for 29 hours with a 10× phase-contrast objective.

Purification of ψ factor

The concentration of Tris in CM was adjusted to 20 mM by the addition of 1.0 M Tris-HCl buffer (pH 7.5). The CM was then loaded on a Q-Sepharose HP column (8×15 mm), which had been equilibrated with 20 mM Tris-HCl (pH 7.5) and equipped with a Gilson HPLC system. After the unbound substances were washed out with the buffer, TSK G3000SW_{XL} (7.8×300 mm, Tosoh, Tokyo, Japan), pre-equilibrated with buffer A (0.05 M sodium phosphate, pH 6.4 containing 0.3 M NaCl), was connected to the outlet of the Q-Sepharose

column. Subsequently, ψ factor was eluted from the combined columns using buffer A as eluent. During these treatments, the flow rate of eluent was 0.4 ml/min. The obtained fractions were assayed for prespore-cell-inducing activity. The active fractions were pooled and used for experiments on the characterization of ψ factor.

SDS-polyacrylamide gel electrophoresis

SDS-PAGE was carried out as described by Laemmli (1970) using 7.5% polyacrylamide gels and the gels were silver stained to show proteins. SDS-PAGE under reducing conditions was done to estimate the purity and the molecular mass of ψ factor. To examine localization of ψ factor activity on SDS polyacrylamide gels, the following experiments were performed. After SDS-PAGE under nonreducing conditions without 2-mercaptoethanol, the gel was cut into 2 mm width slices and each slice was incubated for 20 hours at 22°C in a culture dish containing 1 ml of 20 mM Tris-HCl buffer (pH 7.4). Using this treatment, ψ factor was adsorbed to the substratum of the dish. Then, the gel slices and the buffer were discarded and the biological activity of each dish was measured as in the above mentioned bioassay system.

RESULTS

Prespore-cell-inducing activity of the conditioned medium (CM)

When amoebae of strain V12M2 are incubated in submerged monolayers in the presence of cAMP, they differentiate into prespore and stalk cells in a cell-density-dependent manner (Town et al., 1976; Kay et al., 1978; Kay and Trevan, 1981; Weeks, 1984; Oohata, 1995). In the buffered prespore salt solution (BPSS), most amoebae ultimately differentiate into stalk cells at high cell densities, but at 5×10³ cells/cm² or less, amoebae differentiate into prespore cells without cell contact with the same timing as that of normal development and stalk cell differentiation is scarcely recognized (Oohata, 1995). However, at very low densities (~10² cells/cm²), most cells remain undifferentiated. CM can replace the requirement for high cell density on cell differentiation (Oohata, 1995). Fig. 1 shows the cells incubated in the presence of 50-fold diluted CM at a density of 5×10² cells/cm². Under these conditions, most cells differentiated into prespore cells. In the prespore cell population, small paired prespore cells were occasionally observed (Fig. 1C,F). These cells probably divided during incubation. This will be discussed in a later section.

Prespore cell differentiation at low cell densities depended greatly on incubation temperature as shown in Fig. 2. Moreover, the prespore cell induction rate varied from lot to lot of the culture dishes. Therefore, we chose, for each lot, the incubation temperature at which CM could be assayed most effectively for prespore cell induction (23°C for Fig. 2).

Fig. 3 shows the effect of serial dilutions of CM on prespore cell differentiation. The highest level of differentiation into prespore cells occurred in the presence of 10- to 50-fold diluted CM, with higher concentrations being less effective, confirming that prespore-cell-inducing substance(s) are released into the incubation medium (Weeks, 1984; Oohata, 1995). In contrast, stalk cell differentiation was less than 1% even in the presence of a high concentration of CM (data not shown). This suggests that not enough stalk differentiation-inducing factor, DIF-1 is present in CM for stalk cell differentiation under the conditions adopted in this study.

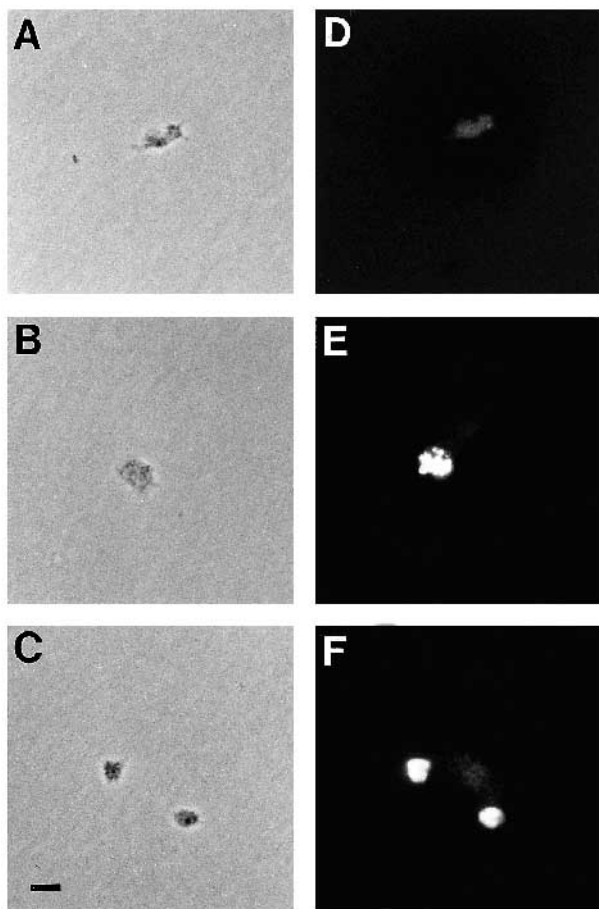


Fig. 1. Immunostaining of cells incubated for 24 hours in BPSS in the presence of 50-fold dilution of CM at a density of 5×10^2 cells/cm². The cells were fixed in situ and stained with FITC-labeled anti-spore serum as described in Materials and Methods. (A-C) Phase contrast; (D-F) PSV staining. (A,D) Undifferentiated cell; (B,E) prespore cell; (C,F) paired prespore cells. Scale bar, 10 μ m.

Separation and purification of prespore-inducing factor(s)

First, to estimate the range of molecular sizes of prespore-inducing factor(s), CM was fractionated with 1×10^4 M_r cutoff ultrafiltration membrane, Ultrafree CL (Millipore, MA, USA) and the prespore-cell-inducing activities of the retained and the dialysable fractions were measured. As shown in Fig. 4A, most of the activity was recovered in the retained fraction. However, some activity was also present in the dialysable fraction, though the maximum induction rate was much lower than that of crude CM (Fig. 4B). The activity of the retained fraction greatly decreased when the fraction was heated at 80°C for 30 minutes but the dialysable fraction was heat-stable. These results indicate that at least two prespore-inducing components, i.e. a heat-labile and high molecular weight factor and a heat-stable and low molecular weight factor, are present in CM. Thus, the retained fraction seems to contain both factors and the dialysable one, only the low molecular weight factor. We termed the high molecular weight factor ψ factor (prespore-inducing factor) and tried to further characterize it. Its activity was also detected in CM of other strains, such as NC-4 and KAx3 (data not shown).

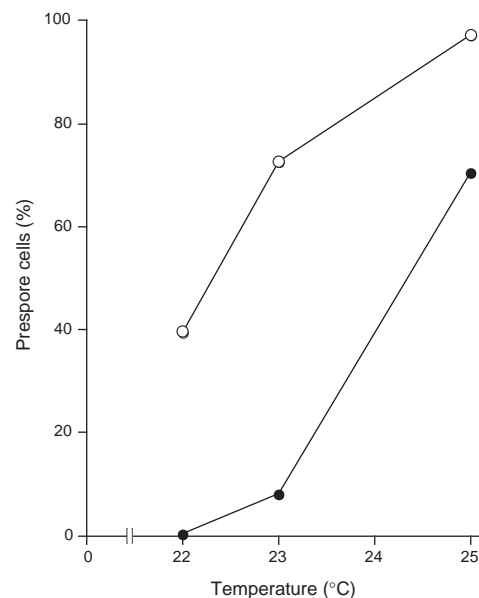


Fig. 2. Temperature dependence of prespore cell differentiation at a density of 10^3 cells/cm² with or without CM. After incubation in BPSS at several temperatures for 24 hours, the cells were fixed and stained, and the percentages of prespore cells were scored microscopically as described in Materials and Methods. Closed circles, control; open circles, 50 μ l of CM was added to 1 ml of medium at the start of incubation.

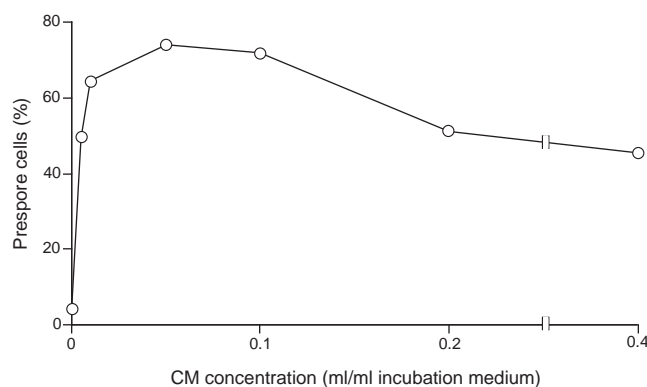


Fig. 3. Dose-response curve for the effect of CM on prespore cell differentiation. Various amounts of CM were added to the medium at the start of incubation.

Purification of ψ factor from CM was carried out using an HPLC system as described in Materials and Methods. In the purification procedure, a single peak of prespore-inducing activity was observed in the high-molecular weight region and no activity was detected in any other fractions. In the active fraction, however, the efficiency of the prespore-cell induction was much lower than that of CM (about 30% of CM) as shown in Fig. 5. These results suggest that both ψ factor and the low molecular weight factor are necessary for prespore cell differentiation and they can be separated from each other by the procedure for purifying ψ factor. To confirm this, the additive effect of ψ factor and the low molecular weight factor was examined as shown in Fig. 6. The induction rate of differentiation of the dialysate and the gel filtration fraction was much lower than

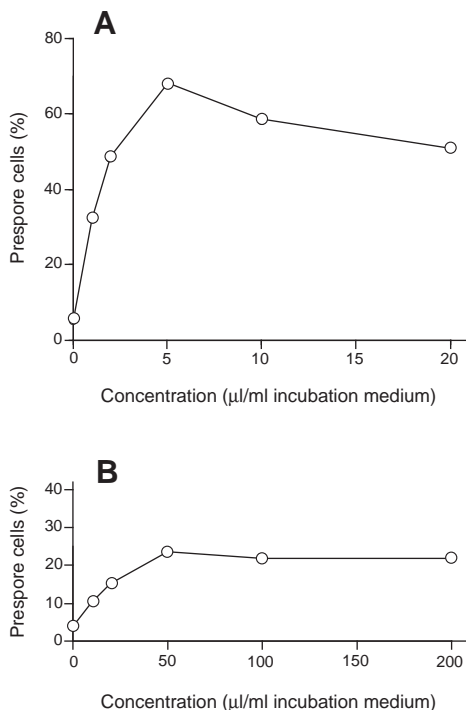


Fig. 4. Dose-response curves for the effect of the retained fraction (A) and the filtrate fraction (B) of ultrafiltration on prespore cell differentiation. CM was concentrated to 1/10 volume using Ultrafree CL (M_r 10^4 cutoff). Various amounts of concentrated fraction (A) or filtrated fraction (B) were added to the medium.

that of the crude CM but both fractions acted synergistically to induce prespore cell differentiation with the same efficiency as that of crude CM. These results indicate that low and high molecular weight factors are both necessary for prespore cell differentiation. Fig. 7 shows the pattern of gel filtration HPLC of ψ factor. The activities were measured in the presence of the dialysate. The relative molecular mass of the factor was determined to be $180 \times 10^3 M_r$. The fractions containing prespore-inducing activity were analyzed by SDS-PAGE under reducing conditions. As shown in Fig. 8 lane b, a major band was recognized at a molecular mass of $106 \times 10^3 M_r$ on a silver-stained gel and the intensity of the band for each fraction obtained by the gel permeation HPLC correlated with the strength of prespore-inducing activity. To confirm that the protein of the major band is ψ factor, the active fractions were loaded on an SDS polyacrylamide gel under non-reducing con-

Fig. 6. Prespore cell induction by crude CM and low molecular and high molecular weight factors. The dialysate is a filtrate of CM that had been sent through M_r 5×10^3 cutoff ultrafiltration membrane. The G3000 fraction was obtained as described in Fig. 5. CM or each factor was added to the incubation medium at the concentration that most efficiently induced prespore cells. Each bar represents the mean \pm s.d. of three independent experiments after subtraction of the control value ($15.7 \pm 5.6\%$).

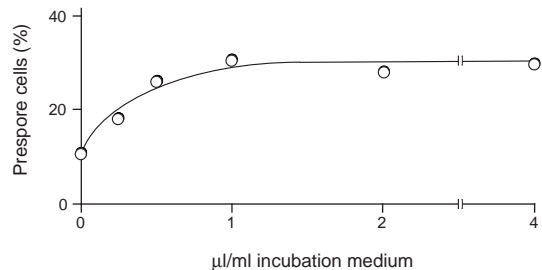
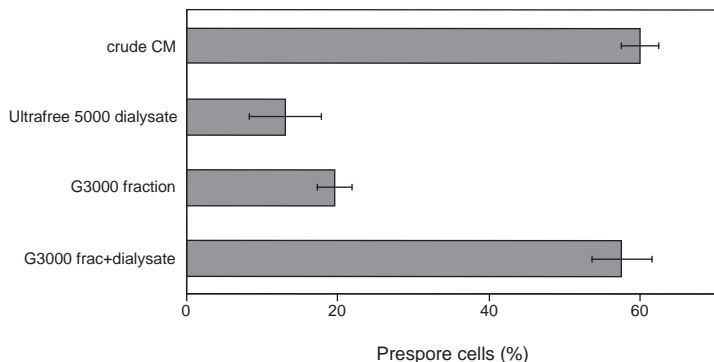


Fig. 5. Dose-response curve for the effect of the gel filtration fraction on prespore cell differentiation. CM was applied to the Q-Sepharose-TSK G3000SWXL column described in Materials and Methods and the fraction with the highest activity was used.

ditions. Then, the gel was cut into 2 mm slices and the activity was measured for each slice as described in Materials and Methods. Under non-reducing conditions, the major band shifted to $88 \times 10^3 M_r$ and the activity was located in the gel slice corresponding to a molecular mass of $88 \times 10^3 M_r$. The protein eluted from the active gel slice was reapplied to an SDS polyacrylamide gel under reducing conditions and gave a single band at a molecular mass of 106×10^3 as shown in Fig. 8 lane c. These results indicate that the major band at a relative molecular mass of 106×10^3 by SDS-PAGE, under reducing conditions, is ψ factor.

Induction of prespore cell division by ψ factor

In the presence of a high concentration of ψ factor, paired prespore cells were observed very frequently, particularly when the incubation temperature was raised. These cells were smaller than the single prespore cells, and generally each set had a similar shape and contained about the same number of PSV (Fig. 2C,F). To examine whether ψ factor induces cell division during prespore cell differentiation, the cells were monitored using time-lapse video during incubation at 25°C . Since amoebae moved very actively during the first 6 hours of incubation, the same cells could not be observed throughout. During this time, however, no cell division was observed with or without ψ factor and the dialysate. After about 6 hours, the cells ceased to move and could then be observed continuously. Fig. 9 shows the frequencies of the cell division during the incubation in the presence and absence of ψ factor and the dialysate. In the control, $75.7 \pm 11.0\%$ ($n=3$) of the amoebae differentiated into prespore cells and 6 of 53 cells divided during 29 hours of incubation. In contrast, in the presence of 500 units



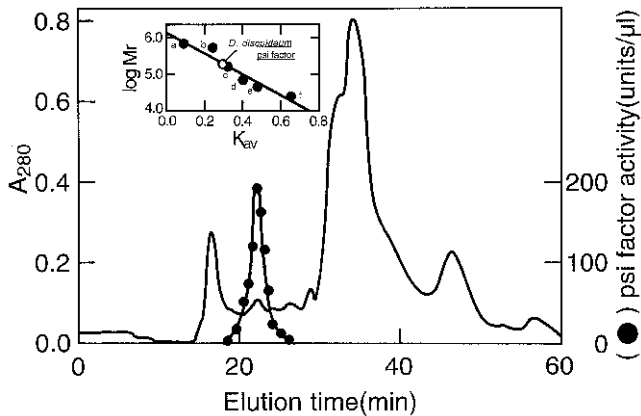


Fig. 7. Elution profile on TSK G3000SW_{XL}. The collected CM was loaded on the Q-Sepharose column, then the substances adsorbed on the Q-Sepharose were eluted into the connected TSK G3000SW_{XL} column. The ψ factor activity was measured by bioassay as described in Materials and Methods. Each value (units) shows the prespore-cell-inducing activity after subtraction of the control value (dialysate only) of 27.6 units. Inset: estimation of molecular mass of native ψ factor by TSK G3000SW_{XL}. The relative molecular mass standard proteins were: (a) thyroglobulin, 670×10^3 ; (b) ferritin, 470×10^3 ; (c) immunoglobulin G, 158×10^3 ; (d) bovine serum albumin, 68×10^3 ; (e) ovalbumin, 44×10^3 ; (f) α -chymotrypsinogen, 24.5×10^3 .

of ψ factor and the dialysate, $94.8 \pm 1.1\%$ ($n=3$) of the amoebae differentiated into prespore cells. Of 46 cells, 41 divided during 8–18 hours of incubation and 21 of the 82 daughter cells divided once more between 11 and 23 hours. In the presence of ψ factor alone, about the same proportion of cells divided as in the presence of the two factors but the dialysate alone induced cell division at the low rate observed in the control (data not shown). At 23°C , cell division was very rare in control cells ($<0.1\%$) but, in the presence of ψ factor, about 30% of the prespore cells were paired cells although a second division was scarcely observed (see Fig. 1). These results indicate ψ factor induces not only prespore cell differentiation but cell division in the course of prespore cell differentiation. Cell division does not appear to be necessary for prespore cell differentiation per se since, in the absence of the factor, most cells differentiate into prespore cells without cell division (Fig. 9A) as reported previously (Kay and Trevan, 1981; Hashimoto et al, 1988). The effect of ψ factor on cell division was specific for prespore cells since, in the presence of ψ factor and 2 nM DIF-1, 90% of cells differentiated into stalk cells but no cell division was observed.

DISCUSSION

In *Dictyostelium discoideum*, cell differentiation only occurs at high cell density (for review see, Schaap, 1991). One important reason for this is that the maintenance of adequate levels of intracellular and extracellular cAMP is essential for development (for reviews, see, Gross, 1994; Parent and Devreotes,

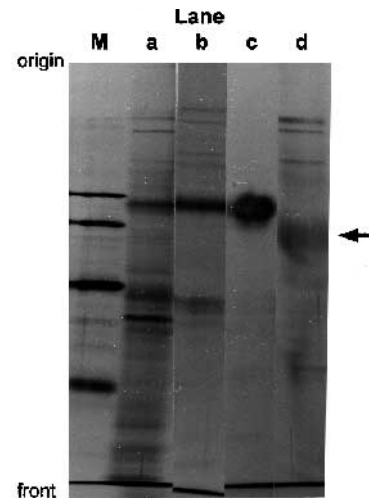


Fig. 8. SDS-PAGE of ψ factor. SDS-PAGE was performed as described in Materials and Methods. Lane M, the molecular weight marker proteins; β -galactosidase (116×10^3), phosphorylase b (97.4×10^3), bovine serum albumin (66.3×10^3) and ovalbumin (43.5×10^3). Lane a, CM ($0.6 \mu\text{g}$ protein). Lane b, active fractions obtained by Q-Sepharose-TSK G3000SW_{XL} chromatography ($0.26 \mu\text{g}$ protein). Lane c, ψ factor extracted from SDS-PAGE without 2-mercaptoethanol (e.g. lane d). Lane d, active fractions obtained by Q-Sepharose-TSK G3000SW_{XL} chromatography ($0.5 \mu\text{g}$ protein). Samples in lanes a–c were reduced with 2-mercaptoethanol and in lane d was non-reduced. The arrow indicates position of the slice having prespore-inducing activity.

1996). Furthermore, several studies have shown that factors in conditioned media mimic high cell density in the presence of cAMP (Town et al., 1976; Kumagai and Okamoto, 1986;

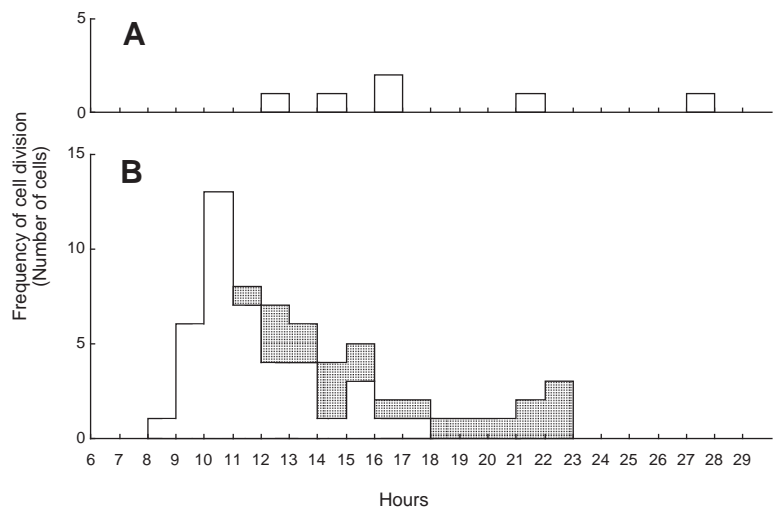


Fig. 9. Quantification of cell division during prespore cell differentiation. Amoebae were incubated at the density of 1×10^3 cells/cm² at 25°C and were monitored for 29 hours as described in Materials and Methods. Each figure is the sum of three independent experiments. (A) The cells were incubated in 1 ml BPSS; 53 cells were monitored. (B) The cells were incubated in 1 ml medium containing 500 units of ψ factor which was partially purified as described in Fig. 5 and $50 \mu\text{l}$ of the dialysate; 46 cells were monitored. Open areas, first division; dotted areas, second division.

Gibson and Hames, 1988; Yamada and Okamoto, 1990; Gomer et al., 1991). With strain V12M2, amoebae differentiate into prespore cells in submerged culture at very low densities ($\sim 10^2$ cells/cm²) if cAMP and adequately diluted CM are provided (Oohata, 1995; Fig. 3). The present study has shown that at least two factors involved in prespore cell differentiation are released into the medium. One is a heat-stable, low molecular weight factor(s) and the other is a heat-labile protein, designated as psi(ψ) factor. ψ factor and the low molecular weight factor(s) synergistically induced prespore cell differentiation of isolated V12M2 amoebae. In contrast, NC-4 amoebae scarcely differentiated under the same conditions but did differentiate into prespore cells when they were incubated at a high cell density (10^6 cells/cm²) for 6 hours before incubation in the presence of the factors (data not shown). Therefore, the factors identified in this study are candidates for prespore-cell-inducing factors common to all *D. discoideum* strains. However, previous reports indicated that amoebae of NC-4 and an axenic strain differentiate in the presence of cAMP and CMF or DCF (differentiation competent factor) at densities of $\sim 10^3$ cells/cm² without addition of any other factor (Gomer et al., 1991, Yamada and Okamoto, 1990). Presumably, this is because, at such densities, enough of ψ factor and the low molecular weight factor(s) are released by the cells themselves for prespore cell differentiation, as is observed in the case of V12M2 (Oohata, 1995).

This purification study has shown that ψ factor is a protein and its molecular mass is 180×10^3 on the gel filtration column. When the active fraction of gel filtration was subjected to SDS-PAGE, the prespore-cell-inducing activity was localized in the $106 \times 10^3 M_r$ band. Therefore, ψ factor appears to form a dimer under the HPLC conditions used. CMF is also a similar molecular mass glycoprotein ($M_r 8 \times 10^4$) (Gomer et al., 1991). However, ψ factor is considered to be a novel factor different from CMF, because the characteristics of ψ factor appear to be different from those of CMF. CMF is heat-stable and its degradation products also have its activity (Gomer et al., 1991). In contrast, ψ factor is heat-labile and no substances of lower molecular weight with its activity have been detected in either CM or HPLC fractions. Moreover, recombinant CMF (provided by Dr Gomer) had no effect on prespore cell induction of V12M2 (data not shown).

CM, in addition to inducing prespore cell differentiation, induced cell division during prespore cell differentiation. The behavior of this cell-division-inducing activity coincided with that of the prespore-cell-inducing activity of the high molecular weight factor not only on Q-Sepharose-G3000 columns but also on other columns, ammonium sulphate fractionation and SDS-PAGE (data not shown). These findings suggest that both activities originate in the same molecule, ψ factor. In animals, many growth factors have been shown to play important roles as both mitogens and morphogens (for review see Massague, 1990; Gospodarowicz, 1990; Cooke, 1991). ψ factor may be such a growth factor and play essential roles in mitosis and differentiation of *Dictyostelium discoideum*. This is the first evidence that molecules for both proliferation and differentiation are ubiquitously involved in the development of eucaryotes and not confined to vertebrates. Previous studies have demonstrated that mitosis occurs during the multicellular stages of *Dictyostelium discoideum* (Bonner and Frascella, 1952; Zada-Hames and Ashworth, 1978) and that cell numbers

increase in these stages (Zada-Hames and Ashworth, 1978; Hashimoto et al., 1988). Moreover, there is much evidence for cell cycle progression during development (Durstun and Vork, 1978; Zimmerman and Weijer, 1993, Araki and Maeda, 1995; Luo et al., 1995). Notably, Durstun and Vork (1978) and Zimmerman and Weijer (1993) have reported that DNA synthesis occurs in prespore cells but not in prestalk cells. Their reports, together with our data on in vitro differentiation, demonstrate that the cell cycle proceeds exclusively in prespore cells. ψ factor probably induces mitosis during the course of prespore cell differentiation in vivo. It has been reported that the cell number in small cell masses (<200 cells) does not increase while they develop to fruiting bodies (Hashimoto et al., 1988). This may be because ψ factor accumulates in a sufficient quantity to induce prespore cells but not to induce cell division in small cell aggregates. In fact, our preliminary experiments indicate that the concentration needed for cell division is higher than that for prespore cell differentiation.

Hitherto, two types of heat-stable and diffusible factors involved in prespore cell differentiation have been identified; one is the spore-protein-inducing factor, SPIF (Gibson and Hames, 1988) and the other is the prespore-inducing factor, PIF (Kumagai and Okamoto, 1986). SPIF is methionine or a methionine derivative and its action can be substituted by methionine (Gibson and Hames, 1988). The dialysable factor of this study does not seem to be SPIF, because 20 μ M methionine had no effect on prespore cell differentiation in our system. PIF is stable in alkali and acid, has a M_r of 150-400 and induces prespore cell differentiation of NC-4 amoebae (Kumagai and Okamoto, 1986). DIF-1 also promotes prespore cell differentiation at a low concentration and cells are considered to accumulate nearly enough DIF-1 for prespore cell induction at the cell densities used in this study (Oohata, 1995). In fact, 0.1 nM DIF-1 occasionally promoted prespore cell induction; however, it could not substitute for the dialysate of CM. Therefore, it is suggested that the dialysate contains, in addition to DIF-1, another prespore-inducing substance, presumably, PIF or a novel factor. Further purification of our dialysable factor is now in progress.

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REFERENCES

- Araki, T. and Maeda, Y. (1995). Cell-cycle progression during the development of *Dictyostelium discoideum* and its relation to the subsequent cell-sorting in the multicellular structures. *Develop. Growth Differ.* **37**, 479-485.
- Berks, M., Traynor, D., Carrin, I., Insall, R. H. and Kay, R. R. (1991). Diffusible signal molecules controlling cell differentiation and patterning in *Dictyostelium*. *Development Supplement* **1**, 131-139.
- Bonner, J. T. (1947). Evidence for the formation of cell aggregates by chemotaxis in the development of the slime mold *Dictyostelium discoideum*. *J. Exp. Zool.* **10**, 1-26.
- Bonner, J. T. and Frascella, E. B. (1952). Mitotic activity in relation to differentiation in the slime mold *Dictyostelium discoideum*. *J. Exp. Zool.* **121**, 561-571.
- Cooke, J. (1991). Inducing factors and the mechanism of body pattern formation in vertebrate embryos. In *Current topics in developmental biology*, vol. 25 (ed. Bode, H. R.), pp. 45-76. San Diego: Academic Press Inc.
- Durstun, A. J. and Vork, F. (1978). The spatial pattern of DNA synthesis in *Dictyostelium discoideum* slugs. *Exp. Cell Res.* **115**, 454-457.

- Early, A. E. and Williams, J. G.** (1988). A *Dictyostelium* prespore-specific gene is transcriptionally repressed by DIF in vitro. *Development* **103**, 519-524.
- Gibson, F. P. and Hames, D.** (1988). Characterization of a spore protein inducing factor from *Dictyostelium discoideum*. *J. Cell Sci.* **89**, 387-395.
- Gomer, R., Yuen, I.S. and Firtel, R. A.** (1991). A secreted $80 \times 10^3 M_r$ protein mediates sensing of cell density and the onset of development in *Dictyostelium*. *Development* **112**, 269-278.
- Gospodarowicz, D.** (1990). Fibroblast growth factor and its involvement in developmental processes. In *Current Topics in Developmental Biology*, vol 24 (ed. Nilsen-Hamilton, M.), pp. 57-95. San Diego: Academic Press, Inc.
- Gross, J.** (1994). Developmental decisions in *Dictyostelium discoideum*. *Microbial Reviews* **58**, 330-351.
- Hashimoto, Y., Nakamura, R., Muroyama, T. and Yamada, T.** (1988). Studies on tiny fruiting bodies of the cellular slime mold, *Dictyostelium discoideum*. *Cytologia* **53**, 337-340.
- Kay, R. R., Garrod, D. and Tilly, R.** (1978). Requirements for cell differentiation in *Dictyostelium discoideum*. *Nature* **271**, 58-60.
- Kay, R. R. and Trevan, D. J.** (1981). *Dictyostelium* amoebae can differentiate into spores without cell-to-cell contact. *J. Embryol. Exp. Morph.* **62**, 369-378.
- Kay, R. R., Berks, M. and Traynor, D.** (1989). Morphogen hunting in *Dictyostelium*. *Development* **1989 Supplement**, 81-90.
- Kopachik, W. J., Dhokia, B. and Kay R. R.** (1985). Selective induction of stalk-cell-specific proteins in *Dictyostelium*. *Differentiation* **28**, 209-216.
- Kumagai, A. and Okamoto, K.** (1986). Prespore-inducing factors in *Dictyostelium discoideum*. Developmental regulation and partial purification. *Differentiation* **31**, 79-84.
- Laemmli, U. K.** (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
- Luo, Q., Michaelis, C. and Weeks, G.** (1995). Cyclin B and Cdc2 expression and Cdk2 kinase activity during *Dictyostelium* differentiation. *DNA & Cell Biol.* **14**, 901-908.
- Massague, J.** (1990). The transforming growth factor- β family. In *Ann. Rev. Cell Biol.* vol. **6** (ed. G. E. Palade, B. M. Albert and J. A. Spudich), pp. 597-641. Palo Alto: Annual Reviews Inc.
- Oohata, A. A.** (1992). Induction of cell differentiation of isolated cells in *Dictyostelium discoideum* by low extracellular pH. *Devel. Growth & Differ.* **34**, 693-697.
- Oohata, A. A.** (1995). Factors controlling prespore cell differentiation in *Dictyostelium discoideum*: Minute amounts of differentiation-inducing factor promote prespore cell differentiation. *Differentiation* **59**, 283-288.
- Parent, C. A. and Devreotes, P. N.** (1996). Molecular genetics of signal transduction in *Dictyostelium*. In *Annu. Rev. Biochem.* vol. **65** (ed. C. C. Rishardson) pp 411-440. Palo Alto: Annual Review Inc.
- Schaap, P.** (1991). Intercellular interactions during *Dictyostelium* development. In *Microbial Cell-Cell Interactions* (ed. M. Dworkin), pp. 147-150. Washington: American Society for Microbiology.
- Town, C. D., Gross, J. D. and Kay, R. R.** (1976). Cell differentiation without morphogenesis in *Dictyostelium discoideum*. *Nature* **262**, 717-719.
- Weeks, G.** (1984). The spore cell induction activity of conditioned media and subcellular fractions of *Dictyostelium discoideum*. *Exp. Cell Res.* **153**, 81-90.
- Williams, J. G.** (1988). The role of diffusible molecules in regulating the cellular differentiation of *Dictyostelium discoideum*. *Development* **103**, 1-16.
- Yamada, Y. and Okamoto, K.** (1990). Three steps in prespore differentiation in *Dictyostelium discoideum* with different requirements of cellular interaction. *Differentiation* **44**, 81-87.
- Zada-Hames, I. M. and Ashworth, J. M.** (1978). The cell cycle and its relationship to development in *Dictyostelium discoideum*. *Dev. Biol.* **63**, 307-320.
- Zimmerman, W. and Weijer, C. J.** (1993). Analysis of cell cycle progression during the development of *Dictyostelium* and its relationship to differentiation. *Dev. Biol.* **160**, 178-185.

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