A prespore-cell-inducing factor in *Dictyostelium discoideum*: its purification and characterization

Manabu NAKAGAWA*, Akiko A. OOHATA†, Hiromasa TOJO‡ and Shigeru FUJII*¹

*Laboratory of Chemistry, Kansai Medical University, 18-89 Uyama-Higashi, Hirakata, 573-1136, Japan, †Laboratory of Biology, Kansai Medical University, 18-89 Uyama-Higashi, Hirakata, 573-1136, Japan, and ‡Department of Molecular Physiological Chemistry, Osaka University Medical School, 2-2 Yamadaoka, Suita, 565-0871, Japan

Under starvation conditions, amoebae of *Dictyostelium discoideum* aggregate to form multicellular masses; the aggregates are then initiated to differentiate. We have reported previously that a signal substance exists in conditioned medium of *D. discoideum*, and we named it prespore-cell-inducing factor (psi, Ψ factor) [Oohata, Nakagawa, Tasaka, and Fujii (1997) Development 124, 2781–2787]. The factor can induce isolated amoebae to differentiate into prespore cells. Moreover, we suggested that it caused not only cell differentiation but also cell division. In the present study, we have purified Ψ factor from the conditioned medium and characterized it. The purified Ψ factor induced both prespore cell differentiation and cell division of prespore cells. Its apparent molecular mass was 180 kDa by gel filtration and 106 kDa by

SDS/PAGE. Based on these results, Ψ factor exists as a dimer in normal conditions. Periodic acid/Schiff staining showed that Ψ factor was a glycoprotein. It was ascertained by Edman degradation that Ψ factor is blocked at the N-terminal. Treatment with pyroglutamate aminopeptidase removed the N-terminal block and allowed determination of the amino-acid sequence of Ψ factor. Moreover, three internal amino-acid sequences were determined in limited proteolysis experiments using trypsin and endoproteinase Lys-C. The homology search for these sequences supports the fact that Ψ factor is a novel differentiation factor.

Key words: *Dictyostelium*, differentiation, growth factor.

INTRODUCTION

The cellular slime mould *Dictyostelium discoideum* is one of the simplest systems for the study of cell differentiation. *D. discoideum* cells normally exist as individual amoebae that feed on bacteria. When the cells overgrow their food supply and become starved, they aggregate, using relayed pulses of cAMP, to form multicellular structures. Cells in the aggregates then differentiate into prespore and prestalk cells, which develop into spore and stalk cells respectively in the subsequent fruiting bodies.

Several factors have been reported to play important roles in differentiation of *D. discoideum*. Differentiation-inducing-factor-1 and conditioned medium factor (CMF) especially have been well characterized. Differentiation-inducing-factor-1 is a low molecular chlorinated hexaphenone which induces prestalk and stalk cell differentiation [1–3]. CMF is a glycoprotein with a molecular mass of 80 kDa and its cleaved products are considered to act as an indicator of cell density in starved NC-4 strain cells and in axenic strains, such as KAx-3, and to be necessary for prespore and prestalk gene expression [4]. At high cell densities ($\sim 10^5$ cells/cm²), the concentration of CMF is sufficient to enable cells to enter the multicellular stage of the developmental cycle, whereas at low cell densities ($\leq 5 \times 10^3$ cells/cm²) cells do not express genes required for early development. However, the V12M2 cells used in this study differentiate into prespore and prestalk cells in a salt solution containing cAMP even at a cell density of 5×10^3 cells/cm², at which cell–cell contact does not exist [5]. Thus strain V12M2 is suitable for investigation of prespore and prestalk cell differentiation, excluding high-cell-density-dependent processes in early development.

In a previous report [6], we showed the presence of a protein which induced prespore cell differentiation in conditioned medium (CM). The protein was named prespore-inducing (psi, Ψ) factor. The previous study [6], using the partially purified factor, suggested that the factor induced not only prespore cell differentiation but also cell division of prespore cells. Thus Ψ factor is considered to play an important role in the development of *D. discoideum*. In the present work, Ψ factor was purified and was characterized as a novel and unique differentiation factor.

EXPERIMENTAL

Materials

Q-Sepharose HP and TSK G3000SW_{XL} were from Pharmacia (Tokyo, Japan) and Tosoh (Tokyo, Japan) respectively. Cosmosil 5C₄-300, Cosmosil 5C₈-300 and poly(vinyl pyrrolidone) (PVP)-40 were products of Nacalai Tesque (Kyoto, Japan). Pyroglutamate aminopeptidase was from Takara (Kyoto, Japan). Tosylphenylalanylchloromethane (TPCK)-treated trypsin and endoproteinase Lys-C were from Worthington Biochemical (Freehold, NJ, U.S.A.) and Boehringer Mannheim (Mannheim, Germany) respectively. The molecular-mass standards BSA, ovalbumin, β -galactosidase, phosphorylase *b*, thyroglobulin, ferritin, IgG and α -chymotrypsinogen, and cAMP were obtained from Sigma (St. Louis, MO, U.S.A.). Ultrafree CL-LCC and Immobilon-P PVDF membrane were from Millipore (Bedford, MA, U.S.A.). Protein was assayed using a protein-assay kit (Bio-Rad, Richmond, CA, U.S.A.). The silver stain kit, 2D silver stain II Daiichi, was prepared by Daiichi Pure Chemicals (Tokyo,

Abbreviations used: Ψ factor, prespore-inducing factor; CM, conditioned medium; CMF, conditioned medium factor; TFA, trifluoroacetic acid; TPCK, tosylphenylalanylchloromethane; CBB, Coomassie Brilliant Blue; PVP, poly(vinyl pyrrolidone); PAS, periodic acid/Schiff.

¹ To whom correspondence should be addressed (e-mail fujii@makino.kmu.ac.jp).

Japan). Culture dishes were purchased from Nunc (Roskilde, Denmark). All other reagents used were of HPLC grade or analytical grade. For the Ψ factor bioassay, FITC-conjugated anti-*D. mucoroides* spore serum was kindly provided by Dr. Okamoto (Kyoto University, Kyoto, Japan).

Culture of *D. discoideum*

Amoebae of *D. discoideum* strain V12M2 were grown with *Klebsiella aerogenes* on a modified SM agar [4.5 g/l KH_2PO_4 , 1.34 g/l K_2HPO_4 , 1.0 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10.0 g/l glucose, 10.0 g/l Bactopeptone (Difco), 1.0 g/l yeast extract (Difco) and 18–20 g/l Bacto-agar (Difco)] at 23.5 °C for 28 h [6].

Production of Ψ factor

Cultured amoebae were washed five times in Bonner's salt solution [7] containing antibiotics (10 mM KCl, 10 mM NaCl, 3 mM CaCl_2 , 200 $\mu\text{g}/\text{ml}$ streptomycin sulphate and 15 $\mu\text{g}/\text{ml}$ tetracycline). The washed amoebae were resuspended in Bonner's salt solution and incubated in 15-cm diam. culture dishes at a density of 1.4×10^6 cells/ cm^2 (8×10^6 cells/ml) at 22–23 °C for 24, 48 or 72 h. After incubation, the CM (supernatant) was collected by centrifugation (2800 g for 20 min) and filtered using a membrane filter (pore size 0.45 μm). The highest specific activity of Ψ factor in the medium was found at 48 h (see Table 1). Thus the medium from 48 h incubations was used as CM for purification of Ψ factor

Purification of Ψ factor by HPLC

Ψ factor was purified using a Gilson HPLC system. The CM collected for purification of Ψ factor was buffered with Tris/HCl adjusted to 20 mM by the addition of 1.0 M Tris/HCl (pH 7.5). In the first step of the purification procedure, CM was applied on to a Q-Sepharose HP column (8 mm $\times$ 15 mm), equilibrated with 20 mM Tris/HCl buffer, pH 7.5, and unbound substances were eluted with the buffer. The flow rate was 0.4 ml/min and column eluates were monitored at A_{280} . Absorbed substances were dissociated from the Q-Sepharose using buffer A (50 mM sodium phosphate, pH 6.4/300 mM NaCl) as eluent. The outlet of the Q-Sepharose column was connected to a TSK G3000SW_{XL} column (7.8 mm $\times$ 300 mm), which had been pre-equilibrated with buffer A, and the desorbed substances were separated and fractions were monitored for Ψ factor activity.

In the second step of the purification procedure, the fractions with Ψ factor activity were subjected to gel permeation chromatography using two TSK G3000SW_{XL} columns (both 7.8 mm $\times$ 300 mm) in tandem equilibrated with buffer A; elution was at a flow rate of 0.1 ml/min in order to separate the proteins more effectively.

The Ψ factor obtained by gel-permeation chromatography was further purified by reverse-phase HPLC on Cosmosil 5C₄-300 (2.1 mm $\times$ 30 mm) equilibrated with 0.1 % (w/v) trifluoroacetic acid (TFA), and the flow rate for injection of a sample was 0.4 ml/min. After the injection, elution was performed at a flow rate of 0.1 ml/min and the concentration of solvent B [0.1 % (w/v) TFA in 2-propanol/acetonitrile/water (4:5:1, by vol.)] was raised by 2 %/min.

SDS/PAGE

Protein eluates from HPLC were separated by SDS/PAGE using the method of Laemmli [8]. Proteins on the gels were detected by

silver staining [9] or Coomassie Brilliant Blue (CBB) R-250 staining. Molecular mass markers were: β -galactosidase (116 kDa), phosphorylase b (97.4 kDa), BSA (66.3 kDa) and ovalbumin (43.5 kDa).

Electroblotting of Ψ factor on to PVDF membrane

After performing SDS/PAGE, Ψ factor was electroblotted from the gel on to an Immobilon P PVDF membrane at 2.6 mA/ cm^2 for 3 h [10]. The membrane was then stained with 0.1 % (w/v) CBB R-250 in 50 % (v/v) methanol for 10 min at room temperature and excess dye was removed with methanol/acetic acid/water (1:2:17, by vol.) for 10 min.

Amino-acid sequence analysis of Ψ factor

The CBB R-250-stained protein spot (2 mm $\times$ 6 mm) of Ψ factor was excised from the PVDF membrane and destained $\times 2$ in 50 % (v/v) methanol for 5 min. Subsequently, the membrane was applied to an amino-acid sequencer (model 477A; Applied Biosystems). Solutions of Ψ factor and its enzymic cleavage fragments, separated by reverse-phase chromatography, were dropped on to Polybrene-coated glass filters and the proteins absorbed on to the filter were also subjected to amino-acid sequencing.

Release of acetyl groups from *N*-acetylserine/*N*-acetylthreonine by TFA treatment

Ψ factor absorbed on to the PVDF membrane was transferred to a test tube (20 mm $\times$ 120 mm) filled with TFA vapour and was incubated at 60 °C for 60 min [11]. After incubation, the membrane was rinsed $\times 2$ in water and applied to an amino-acid sequencer.

Release of formyl groups by HCl

The membrane with Ψ factor was incubated in 0.6 M HCl for 24 h at 25 °C [12]. After incubation, the membrane was washed in water and subjected to amino-acid sequencing.

Removal of pyroglutamate by pyroglutamate aminopeptidase

Using pyroglutamate aminopeptidase, an attempt was made to deblock the N-terminus of Ψ factor. The membrane electroblotted Ψ factor was washed $\times 2$ in 50 % (v/v) methanol and the membrane was blocked by the addition of 0.5 ml 0.5 % (w/v) PVP-40 in 0.1 % (v/v) acetic acid. The blocking solution was removed and 2 m-units of pyroglutamate aminopeptidase in 0.2 ml of 50 mM sodium phosphate buffer (pH 7.0) containing 10 mM dithiothreitol and 1 mM EDTA was added. After incubation at 37 °C for 12 h, a further 2 m-units of pyroglutamate aminopeptidase was added and incubation was continued for 12 h. The treated membrane was rinsed $\times 2$ in water and was subjected to amino-acid sequencing [13,14].

Limited proteolysis of Ψ factor

In order to obtain information on the internal amino-acid sequence, Ψ factor was treated with TPCK-treated trypsin and endoproteinase Lys-C. The limited proteolysis of Ψ factor with trypsin was carried out as follows. Ψ factor (5.0 μg) was separated by SDS/PAGE and electroblotted on to a PVDF membrane. After electroblotting, Ψ factor was stained with CBB R-250 and the Ψ factor protein spot was excised with a razor blade. The

membrane portion was destained in 50% methanol, rinsed in water and incubated in 0.5% (w/v) PVP-40 in 100 mM acetic acid at 37 °C for 30 min to prevent adsorption of trypsin to the membrane. Excess PVP-40 was removed by washing in water [15]. The membrane portion was then soaked in 0.3 ml of 0.2 M Tris/HCl, pH 8.5/acetonitrile (9:1, v/v) containing 5 µg of TPCK-treated trypsin, and was kept at 37 °C for 12 h. A further 5 µg of trypsin was added and proteolysis was continued for 12 h. Ψ factor (11.6 µg) was also digested with 0.5 µg of endoproteinase Lys-C in 40 µl of 50 mM Tris/HCl, pH 8.5/1 mM EDTA at 37 °C for 15 h [16]. After the limited proteolysis with trypsin and endoproteinase Lys-C, the resultant peptide fragments were absorbed on to Cosmosil 5C₈-300 (2.1 mm × 30 mm column), equilibrated with 0.1% TFA, at a flow rate of 0.4 ml/min. The fragments were eluted with a gradient of solvent C [0.09% TFA in water/acetonitrile (1:19, v/v)], the concentration of which was raised from 0–40% in the first 60 min and from 40–70% in the next 30 min. The flow rate was 0.1 ml/min and the eluates were monitored at A₂₁₄ [17]. The separated peptide fragments were analysed by amino-acid sequencing (Applied Biosystems 477A).

Detection of Ψ factor glycoprotein by periodic acid/Schiff (PAS) staining

The presence of carbohydrate chain(s) in Ψ factor was determined by PAS staining [18]. Following SDS/PAGE, the gel was divided into two parts using a razor and one part was stained with CBB and the other was soaked in 7.5% (w/v) acetic acid to immobilize proteins in the gel. This section of the gel was then incubated for 45 min at 4 °C in 1% (w/v) periodic acid, and for 45 min at 4 °C in Schiff's reagent. Finally, excess dye was removed by washing the gel in 7% (v/v) acetic acid at room temperature.

Amino-acid analysis of Ψ factor

Ψ factor was absorbed on to Polybrene-coated filter and hydrolysed in 6 M HCl at 120 °C for 24 h using a Pico-Tag Workstation [19]. HCl was removed by evaporation, the hydrolysate was dissolved in 0.2 ml of 0.02 M HCl and 0.1 ml of the solution was loaded on to an amino-acid analyser (Hitachi, model L-8500).

Measurement of Ψ factor activity and cell division

We estimated the cell differentiation activity of Ψ factor by bioassay as described previously [6]. Prespore cells were detected by the fluorescence of FITC-conjugated anti-*D. mucoroides* spore serum binding to prespore specific vacuoles. The activity was estimated as the ratio of differentiated cells to total cells. In the present study, a unit of Ψ factor activity is defined as that amount which induces the formation of 1% prespore cells in the standard assay [6]. In the prespore cell population, small paired prespore cells were frequently observed. Previously, we confirmed that each paired prespore cell originated from a divided cell and that non-differentiated cells did not divide during incubation [6]. Therefore, in the present study, paired prespore cells were estimated as divided cells.

Protein assay

Protein concentrations were estimated by the method of Bradford [20] using a Bio-Rad protein assay kit, and with BSA as a standard.

RESULTS

Production of Ψ factor

In the present study, strain V12M2 was used because this strain showed higher Ψ factor activity in CM than other strains, such as NC-4 and KAx-3. To obtain CM with a high specific activity of Ψ factor, cells were incubated in a simple salt solution as submerged monolayers at the relatively low cell density of 1.4×10^6 cells/cm². Under these conditions, cell lysis was rarely observed and most proteins contained in the CM were secreted proteins. To determine an optimum incubation time for purification of Ψ factor from the culture medium, cells were cultivated for 24 h, 48 h and 72 h, the culture medium was collected, filtered and Ψ factor activity and total protein in the medium were measured. The highest specific activity of Ψ factor in the medium was found at 48 h (Table 1). Thus the medium from 48 h incubations was used as CM for purification of Ψ factor.

Purification of Ψ factor

Unbound material was eluted from the Q-Sepharose column (Figure 1a) and no Ψ factor activity was detected. When bound material was dissociated from Q-Sepharose and subjected to gel permeation on TSK G3000SW_{XL}, prespore-inducing activity was present in the eluted fractions. The activity was observed only in the high-molecular mass region (Figure 1b). The active fractions were then applied to two TSK G3000SW_{XL} columns in tandem, as described in the Experimental section. The elution profile is shown in Figure 2. The chromatogram showed that the peak of Ψ factor activity was identical with the main peak monitored at A₂₈₀. The fractions from the second purification step having the highest Ψ activity were collected and an aliquot of the pooled fractions was applied on to a Cosmosil 5C₄-300 column. The elution profile is shown in Figure 3; protein and Ψ activity were detected only in the fraction eluting at 25.8 min. The results of the reverse-phase chromatography suggest that the pooled solution contained a single protein. To verify the purity of Ψ factor from the purification steps, SDS/PAGE was carried out (Figure 4). As shown in Figure 4 (lane b), in the first purification step, several proteins were isolated and one protein, at a molecular mass of 106 kDa on the silver-stained gel, was selectively concentrated. The protein was purified to a single band in the next purification step (Figure 4, lane c). When SDS/PAGE was done under non-reducing conditions, without 2-mercaptoethanol, the protein band was shifted to a molecular mass of 88 kDa and a gel slice at this position showed Ψ activity (results not shown). These results indicated that Ψ factor was purified in these procedures, which are summarized in Table 2.

Measurement of molecular mass

The molecular mass of Ψ factor was estimated to be 180 kDa by gel filtration (Figure 2, inset) and 106 kDa from the mobility on SDS-polyacrylamide gels (Figure 4). These results indicate that Ψ factor exists as a dimer under normal conditions.

Detection of carbohydrates

After Ψ factor was electrophoresed on SDS-polyacrylamide gel, the gel was split into two parts using a razor. As shown in Figure 5, one part was stained with CBB R-250 (lane a), and the other (lane b), stained with PAS, showed that the Ψ factor stained

Table 1 Ψ factor activity in CM

CM was collected and assayed for Ψ factor activity as described in the Experimental section.

Time of harvest (h)	Protein ($\mu\text{g/ml}$)	Activity (units/ml)	Specific activity (units/mg of protein)
24	13.52	2.6×10^3	192.3×10^3
48	21.32	5.0×10^3	234.5×10^3
72	29.51	5.6×10^3	189.8×10^3

Figure 1 Q-Sepharose-TSK G3000SW_{XL} chromatography (step 1)

(a) CM was applied to a Q-Sepharose HP column (8 mm $\times$ 15 mm) in two injections, each of 10 ml, at 0 and 60 min (shown by arrows). The eluent flow rate was 0.4 ml/min. The eluate was monitored at A_{280} . (b) Elution profile of Q-Sepharose-TSK G3000SW_{XL} chromatography. A TSK G3000SW_{XL} (7.8 mm $\times$ 300 mm) column was connected to the outlet of the Q-Sepharose column. Substances were desorbed from the Q-Sepharose by elution with buffer A (see the Experimental section) and were passed through the TSK G3000SW_{XL} column at a flow rate of 0.4 ml/min; the volume of each fraction collected was 0.4 ml. Ψ factor activity (●) of the fractions was measured by bioassay and the fractions between the two arrows were collected.

positively with the PAS reaction. The result reveals that Ψ factor is a glycoprotein.

Amino-acid analysis of Ψ factor

Analysis of amino-acid composition of Ψ factor was carried out as described in the Experimental section. The results are summarized in Table 3. Methionine residues were not detected and proline residues made up $> 10\%$ of the total amino acids.

N-terminal amino-acid analysis of Ψ factor

Amino-acid analysis of the intact, purified Ψ factor using the automated Edman method gave no information for the N-terminal sequence. This result suggested that the N-terminal was blocked. Three different deblocking methods were tried, as described in the Experimental section. Treatment of the Ψ factor

Figure 2 Elution profiles of protein and Ψ factor from tandem TSK G3000SW_{XL} column chromatography (step 2)

The purified Ψ factor collected from step 1 of the purification procedure was subjected to gel-permeation chromatography through two TSK G3000SW_{XL} columns in tandem. The flow rate through the columns was 0.1 ml/min and the volume of each fraction collected was 0.2 ml. Ψ factor activity (●) of the fractions was measured by bioassay and the fractions between the arrows were collected. Inset: estimation of molecular mass of native Ψ factor after TSK G3000SW_{XL} chromatography. The molecular-mass standards were: (a) thyroglobulin, 670 kDa; (b) ferritin, 470 kDa; (c) IgG, 158 kDa; (d) BSA, 68 kDa; (e) ovalbumin, 44 kDa; (f) α -chymotrypsinogen, 24.5 kDa.

Figure 3 Reverse-phase HPLC of Ψ factor

The purified Ψ factor collected from step 2 of the purification procedure (1.30 μg) was applied to a Cosmosil 5C₄-300 reverse-phase column (2.1 mm $\times$ 30 mm) as described in the Experimental section. The protein was eluted with a linear gradient of solvent B [0.1% TFA in 2-propanol/acetonitrile/water (4:5:1, by vol.)] at 2%/min, from 0–100% in 50 min, at a flow rate of 0.1 ml/min. The A_{280} of the fractions was monitored.

with TFA or 0.6 M HCl did not allow N-terminal sequencing by Edman degradation. These results indicated that the N-terminal residue was neither formylated nor N-acetylated. Digestion of Ψ

Figure 4 SDS/PAGE of Ψ factor from the purification steps

Lane a, CM (500 ng protein); lane b, proteins from Q-Sepharose-TSK G3000SW_{XL} chromatography (50 ng applied); lane c, protein from tandem TSK G3000SW_{XL} columns (50 ng applied). The molecular-mass standards were: β -galactosidase, 116 kDa; phosphorylase b, 97.4 kDa; BSA, 66.3 kDa; ovalbumin, 43.5 kDa.

factor with pyroglutamate aminopeptidase removed a blocking group and showed that the adjacent residue to the N-terminus was threonine. Based on this fact, the N-terminus was determined to be pyroglutamate. The N-terminus and the adjacent sequence was (< Q)TPSTHLD, where < Q is pyroglutamate.

Internal amino-acid sequence analysis of Ψ factor

In order to obtain more information on the amino-acid sequence of Ψ factor, Ψ factor was digested with TPCK-treated trypsin and endoproteinase Lys-C. The trypsin-treated Ψ factor was applied to a Cosmosil 5C₈-300 column and the elution profile is shown in Figure 6. Four fractions, corresponding to peaks 1, 2, 3 and 4, were collected and applied to the amino-acid sequencer. Based on analysis of the peak 1 fraction, an amino-acid sequence of 28 residues was determined, as shown in Table 4. No

Figure 5 SDS/PAGE of the purified Ψ factor

Lane a, purified Ψ factor (0.89 μ g protein) stained with CBB R-250; lane b, purified Ψ factor (0.89 μ g protein) stained with PAS reaction. The molecular-mass standards were: β -galactosidase, 116 kDa; phosphorylase b, 97.4 kDa; BSA, 66.3 kDa.

information on the amino-acid sequence of Ψ factor was obtained from fractions of the other three peaks; peaks 2 and 3 contained autolytic fragments of trypsin itself and peak 4 contained several peptide fragments. In the limited proteolysis experiment of Ψ factor with endoproteinase Lys-C described in the Experimental section, six fractions were collected. Two fractions gave information on the internal amino-acid sequences of Ψ factor and were composed of 14 and six residues respectively (Table 4). No sequences were obtained from the other four fractions, as they contained several peptide fragments. The internal amino-acid sequences of Ψ factor are summarized in Table 4.

Measurement of cell differentiation and cell division

Our previous study, using partially purified Ψ factor, suggested that Ψ factor apparently induced not only prespore cell differentiation but also cell division of prespore cells. In the present study, the dose-response of the effect of purified Ψ factor on prespore cell differentiation and cell division was examined. The results are shown in Figure 7. In our assay system, 10 ng of Ψ factor was required to differentiate a half of the maximum

Table 2 Purification of Ψ factor

Ψ factor collected from CM was subjected to gel-permeation chromatography through Q-Sepharose-TSK G3000SW_{XL} and two TSK G3000SW_{XL} columns in tandem, as described in the Experimental section.

	Volume (ml)	Total protein (μ g)	Total activity (unit)	Specific activity (unit/mg of protein)	Purification (fold)	Yield (%)
Conditioned medium	43.6	575.7	240×10^3	417×10^3	1	100
Q-Sepharose-TSK G3000SW _{XL}	1.90	20.8	81.3×10^3	3909×10^3	9.37	33.9
2x TSK G3000SW _{XL}	5.21	6.8	46.4×10^3	6854×10^3	16.4	19.3

Table 3 Amino-acid composition of Ψ factor from *D. discoideum*

Ψ factor was hydrolysed in 6 M HCl at 120 °C for 24 h. ND, not determined.

Amino-acid residue	Occurrence in Ψ factor (%)
Asx	14.7
Thr	13.3
Ser	6.7
Glx	9.8
Gly	7.6
Ala	1.8
Cys	ND
Val	5.7
Met	0.0
Ile	3.6
Leu	6.4
Tyr	3.6
Phe	4.8
His	2.0
Lys	6.0
Trp	ND
Arg	2.2
Pro	10.8

Figure 6 Reverse-phase HPLC of peptide fragments after trypsin digestion of Ψ factor

To Ψ factor digests (300 μ l) was added 300 μ l of 10% acetonitrile and the sample was applied to a Cosmosil 5C₈-300 reverse-phase column equilibrated with 0.1% TFA. Peptides were eluted with a linear gradient of solvent C [0.09% TFA in water/acetonitrile (1:19, v/v)], from 0–40% in 60 min and from 40–70% in the next 30 min at a flow rate of 0.1 ml/min. The eluate was monitored at A_{214} and the fractions comprising peaks 1, 2, 3 and 4 were collected and subjected to amino-acid sequencing.

Table 4 Internal amino-acid sequences of Ψ factor fragments

Proteolysis	Amino-acid sequence
TPCK-treated trypsin	TASGNYIYDNDFFFPIDYEGFDTDPANR (28 amino acids)
Endoproteinase Lys-C	LVQTTLGSDGLPVL (14 amino acids)
	YFFAPN (six amino acids)

Figure 7 Effects of Ψ factor on prespore cell differentiation and cell division

Various quantities of purified Ψ factor were added to the bioassay system and prespore-cell-inducing activity (○) and cell-division activity (●) were measured as described in the Experimental section. Cell-division activity is expressed as the percentage of paired prespore cells in total prespore cells.

differentiated cells. For cell division, several times more Ψ factor was required than for cell differentiation. These results lead to the conclusion that Ψ factor induces both prespore cell differentiation and cell division of prespore cells.

DISCUSSION

In the life cycle of *D. discoideum*, Ψ factor is considered to play an important part in prespore cell differentiation, as reported previously [6]. To elucidate the function of the factor, an attempt was made to purify and characterize Ψ factor. The conditions for the production of Ψ factor were carefully controlled to obtain conditioned medium (CM) with minimal contamination. Since only limited kinds of proteins were present in the initial CM, purified Ψ factor was obtained by a simple method (only two steps).

The molecular mass of the native Ψ factor was estimated to be 180 kDa by gel permeation chromatography (Figure 2). On SDS-polyacrylamide gels, Ψ factor was observed as a single band with a molecular mass of 106 kDa under reducing conditions and of 88 kDa under non-reducing conditions. These results suggest that Ψ factor exists as a dimer consisting of two identical subunits and that there is no disulphide bridge between the subunits. Ψ factor was also detected on SDS-polyacrylamide gels by PAS staining (Figure 5). When Ψ factor was applied on to a Con A–Sephacrose column, it was strongly bound on to the column and was eluted with a high concentration of methyl- α -D-mannoside (results not shown). These results indicated that Ψ factor is a glycoprotein with asparagine-linked carbohydrates. The structures and functions of the sugar chains are under investigation. The estimated molecular mass of Ψ factor was large compared with the other known proteinaceous differentiation factor of *D. discoideum* [4] or mammalian growth factors like platelet-derived growth factor (PDGF) [21] or leukaemia-inhibitory factor (LIF) [22]. The carbohydrate chains in the molecule may lead to overestimation of the molecular mass of Ψ factor. At the present time, the complete amino-acid sequence is being analysed from the cDNA and its molecular mass will be determined on the basis of the sequence.

The amino-acid composition of the factor is summarized in Table 3. Remarkably, of the total amino acids contained within Ψ factor more than 10% were proline residues. As proline

residues have a characteristic effect on protein tertiary structure, the structure of Ψ factor may be unique. Methionine residues were not detected in Ψ factor by amino-acid analysis, and this is consistent with the result of the N-terminal analysis. It was not possible to determine the N-terminal residue of the native Ψ factor by Edman degradation but, after treatment with pyroglutamate aminopeptidase, the N-terminal residue was found to be pyroglutamate by the Edman method and the remaining N-terminal sequence was shown to be TPSTHLD. These results indicated that the N-terminus of Ψ factor was blocked by pyroglutamate and that an N-terminal peptide having a methionyl residue as an initiating residue was removed from the nascent Ψ factor. Moreover, several peptide fragments were isolated from the trypsin- and endoproteinase Lys-C-digested factor (Figure 6), and their amino-acid sequences were determined (Table 4). In a homology search using the BLAST program [23] at D. D. B. J. (National Institute of Genetics, Shizuoka 411, Japan), the N-terminal amino-acid sequence (< Q)TPSTHLD and the three internal amino-acid sequences (Table 5) did not match any of the registered protein sequences or cDNA sequences. These results support the fact that Ψ factor of *D. discoideum* is a novel and unique protein.

We suggested in our previous paper [6] that Ψ factor in CM had not only prespore-cell-inducing activity, but also cell-division activity. In the present study, we have confirmed that both activities exist in the same molecule (Figure 7). If the molecular mass is 180 kDa, based on estimation by gel permeation chromatography, about 55 pM of Ψ factor was required to differentiate a half of maximal differentiated cells under our conditions (Figure 7). The value is similar to that of other differentiation factors or growth factors [21,24]. Of interest to us was whether the same site in Ψ factor regulated the two events (differentiation and cell division) in sequence, or if separate sites regulated these events independently. To elucidate the mechanism of prespore cell differentiation in *D. discoideum*, it is important to find out the receptor for Ψ factor and then define the biochemical events after binding of Ψ factor to its receptor. Studies along these lines,

including determination of the complete amino-acid sequence by cDNA analysis and the structures of the sugar chains, are in progress.

REFERENCES

- Kopachik, W. J., Dhokia, B. and Kay, R. R. (1985) *Differentiation* **28**, 209–216
- Early, A. E. and Williams, J. G. (1988) *Development* **103**, 519–524
- Kay, R. R., Berks, M. and Traynor, D. (1989) *Development (Suppl.)*, 81–90
- Gomer, R., Yuen, I. S. and Firtel, R. A. (1991) *Development* **112**, 269–278
- Oohata, A. A. (1992) *Develop. Growth Differ.* **34**, 693–697
- Oohata, A. A., Nakagawa, M., Tasaka, M. and Fujii, S. (1997) *Development* **124**, 2781–2787
- Bonner, J. T. (1947) *J. Exp. Zool.* **10**, 1–26
- Laemmli, U.K. (1970) *Nature (London)* **227**, 680–685
- Ohsawa, K. and Ebata, N. (1983) *Anal. Biochem.* **135**, 409–415
- Matsudaira, P. (1987) *J. Biol. Chem.* **262**, 10035–10038
- Wellner, D., Panneerselvam, C. and Horecker, B. L. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1947–1949
- Ikeuchi, M. and Inoue, Y. (1988) *FEBS Lett.* **241**, 99–104
- Hirano, H., Komatsu, S., Nakamura, A., Kikuchi, F., Kajiwarra, H., Tsunasawa, S. and Sakiyama, F. (1991) *Theor. Appl. Genet.* **83**, 153–158
- Shimada, Y., Sugiyama, A., Tominaga, Y., Iizumi, T. and Tsunasawa, S. (1989) *J. Biochem. (Tokyo)* **106**, 383–388
- Hirano, H., Komatsu, S., Takakura, H., Sakiyama, F. and Tsunasawa, S. (1992) *J. Biochem. (Tokyo)* **111**, 754–757
- Jekel, P. A., Weijer, W. J. and Beintema, J. J. (1983) *Anal. Biochem.* **134**, 347–354
- Ono, T., Tojo, H., Kuramitsu, S., Kagamiyama, H. and Okamoto, M. (1988) *J. Biol. Chem.* **263**, 5732–5738
- Zacharius, R. M., Zell, T. E., Morrison, J. H. and Woodlock, J. J. (1969) *Anal. Biochem.* **30**, 148–152
- Tojo, H., Ono, T., Kuramitsu, S., Kagamiyama, H. and Okamoto, M. (1988) *J. Biol. Chem.* **263**, 5724–5731
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Miyazono, K. and Takaku, F. (1989) *Blood Rev.* **3**, 269–276
- Gough, N. M., Gearing, D. P., King, J. A., Wilson, T. A., Hilton, D. J., Nicola, N. A. and Metcalf, D. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2623–2627
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990) *J. Mol. Biol.* **215**, 403–410
- Assolian, R. K. (1987) *Methods Enzymol.* **146**, 153–163