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Novel Development Rescuing Factors (DRFs) Secreted by the Developing *Dictyostelium* Cells, That are Involved in the Restoration of a Mutant Lacking MAP-kinase ERK2

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ABSTRACT—We found novel development rescuing factors (DRFs) secreted from developing *Dictyostelium* cells, by using a mutant (*erkB*$^-$) which is missing MAP-kinase ERK2 as a test strain for bioassay. The mutant *erkB*$^-$ fails to undergo multicellular morphogenesis due to impaired cAMP signaling. However, such developmental defect can be restored by the presence of low-molecular weight DRFs that are secreted from developing wild-type cells. We previously showed that DIF-1 (Differentiation-Inducing Factor 1 for stalk cells) possesses this activity, indicating a newly discovered role of DIF-1. Surprisingly, however, the mutant *dmtA*$^-$, which is incapable of DIF-1 synthesis still exerts a strong inducing activity of the multicellular morphogenesis of *erkB*$^-$. After analysis of HPLC fractions of conditioned media prepared from both wild type Ax2 and *dmtA*$^-$ strains revealed that both strains secrete at least two novel DRF activities with DIF-like mobility. However, these activities were not derived from other DIFs such as DIF-2 and DIF-3. Identification of these DRFs found in this study would provide insight into the mechanism by which the development of the *erkB*$^-$ mutant is restored and how these factors act in the normal development of *Dictyostelium*.

Key words: Intercellular communication, DIF-1, MAP-kinase ERK2, *dmtA*, *Dictyostelium*

INTRODUCTION

Intercellular communication mediated through secreted factors is essential for both the cell differentiation and morphogenesis of multicellular organisms. *Dictyostelium,* one of the simplest multicellular eukaryotes, provides a model system for studying the mechanisms underlying such intercellular communication. Upon starvation, free-living *Dictyostelium* amoebae initiate multicellular development via their aggregation to form a hemispherical mound where prestalk- and prespore-cell differentiation occurs. These cells are sorted eventually to form a fruiting body consisting of a round spore mass supported by a cellular stalk. Moreover, the proportion and sorted pattern of prestalk and prespore cells are highly regulated. Co-ordinate cell movement and regulated pattern of cell differentiation during morphogenesis suggest that intercellular signaling plays crucial roles in the morphogenesis of *Dictyostelium*.

Most studied extracellular signaling molecules in *Dictyostelium* are cAMP and DIF-1 (Differentiation Inducing Factor for stalk cells). cAMP acts as a chemoattractant for the aggregation and the morphogenetic movement of *Dictyostelium* cells and also regulates gene expressions for the cell differentiation (Konijn *et al*., 1969; Gross *et al*., 1976; Tomchik and Devreotes, 1981; Rietdorf *et al*., 1998). Several mutants with a defect in cAMP signaling have been isolated in *Dictyostelium*. They are all arrested at the preaggregation stage, however, some mutations lacking adenylyl cyclase, cytosolic regulator of adenylyl cyclase or MAP-kinase ERK2 are non-autonomous, because the mutants develop into fruiting bodies in the presence of wild-type cells (Pitt *et al*., 1992; Insall *et al*., 1994; Segall *et al*., 1995). In this connection, we further analyzed the effects of wild type cells on *erkB*$^-$ lacking functional ERK2 and found that the mutant phenotype was not restored by cAMP (Maeda and Kuwayama, 2000). However, certain factors secreted by the developing wild type cells restored developmental defect in the mutant (Maeda and Kuwayama, 2000; Kuwayama *et al*., 2000). This suggests that wild-type cells provide certain signaling molecules other than cAMP that are lacking in the mutant. These signaling molecules were named DRFs

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(Development Rescuing Factors) after their biological activity as mentioned above.

DIF-1 is another well-analyzed signaling molecule in *Dictyostelium*, which is a dichloro-alkylphenone, a hydrophobic signaling molecule that induces differentiation of stalk cells *in vitro* (Town *et al*., 1976; Town and Stanford, 1979; Morris *et al*., 1987). Two DIF-1 related compounds, DIF-2 and DIF-3, were also identified (Morris *et al*., 1988). DIF-3 is the initial product in the breakdown process of DIF-1. DIF-2 has a quite similar structure to DIF-1, which could be a side-product of DIF-1 biosynthesis. DIF-1 accounts for more than 95% of the total bioactivity and DIF-2 for most of the rest (Kay *et al*., 1983; Brookman *et al*., 1987). Both DIF-1 and DIF-2 are also involved in the light scattering responses of early developing cells in suspension, suggesting that they play a distinct role from stalk cell inducers (Wurster and Kay, 1990). Furthermore, we revealed that DIF-1 acts as DRF which induces the multicellular development of *erkB*$^-$ mutant missing MAP kinase ERK2, a component of cAMP signaling cascades in *Dictyostelium* (Segall *et al*., 1995; Kuwayama *et al*., 2000). This is a newly discovered role of DIF-1.

In the present study, we examined whether DIF-1 is a sole agent acting to induce the multicellular development of *erkB*$^-$ mutant. First we assessed whether DIF-2 and DIF-3 also act as DIF-1 did. Second we examined whether a DIF-less mutant (*dmtA*$^-$) that was generated by disrupting the gene encoding methyltransferase which catalyzes the final step of the pathway for the biosynthesis of DIF-1 (Kay, 1998; Thompson & Kay, 2000) secretes certain factors to induce the multicellular development of *erkB*$^-$ mutant. We show here that there are at least two novel hydrophobic factors which are able to induce the multicellular development of *erkB*$^-$ mutant and secreted from both wild type Ax2 and a *dmtA*$^-$ mutant. Identification and further analysis of these factors would provide insight into the mechanism by which the developmental defect of the *erkB*$^-$ mutant is corrected and how these DRFs act in the normal development of *Dictyostelium*.

MATERIALS AND METHODS

Strains and cultures

Dictyostelium discoideum Ax2 (a wild-type strain), HS175 (*erkB*$^-$ mutant), *dmtA*$^-$ (DIF-less mutant) and V12M2 were used. All strains except V12M2 were grown at 21°C in HL5 supplemented with 5 ng/ml of Vitamin B_{12} and 100 ng/ml of folic acid (Watts & Ashworth, 1970). V12M2 cells were grown on SM agar plates in association with *Klebsiera aerogenes* and used for DIF assay.

Spot test for rescue assay

DRF activities were assayed by the spot test. Cells were harvested at 2~6×10^6 cells/ml and were washed twice with 12 mM Na_2K phosphate buffer, pH 6.1 (PB). Then, the washed cells were resuspended in PB at a density of 5×10^7~1×10^8 cells/ml. To examine the effects of DIF-1, DIF-2 and DIF-3, a drop of a 5 μl aliquot of the *erkB*$^-$ cell suspension was spotted on 1.6% hydrophobic agar containing various concentrations. Hydrophobic agar was prepared by extensively washing agar powder with deionized water. To examine the DRF activity of the secreted factors of *dmtA*$^-$, a 5 μl aliquot of the *dmtA*$^-$ cell suspension was placed at the side of a drop of *erkB*$^-$ suspension. The distance between the two spots was about 2 mm. The DRF activities of HPLC fractions were tested in the same way. A 0.5 μl aliquot of each fraction and sometimes 5, 10, 50, 100, 200, and 300-folds dilutions were deposited instead of the *dmtA*$^-$ cell suspension.

Extraction of factors sereted from Dictyostelium cells

Secreted factors were collected and extracted by the same method used for DIF collection and extraction (Kay *et al*., 1983). Washed Ax2 or *dmtA*$^-$ cells were allowed to develop at a density of 1.5×10^7 cells/cm^2 on a layer of cellophane (325P, British Cellophane Co, Bridgewater, UK) supported by a stainless steel mesh in a surgical tray. The underside of the cellophane was bathed with a medium containing 10 mM KCl, 2 mM NaCl, 1mM $CaCl_2$ and 5–10 g/l washed Amberlite XAD-2 resin (BDH). The trays were shaken at about 16 rev./min on a New Brunswick G2 orbital shaker. After 3–5 days, XAD-2 beads whose color had changed to yellow from adsorbing material were harvested and washed with water. The adsorbed material was eluted with ethanol. The elute concentrated by rotary evaporation, was dispersed in water, and the process was repeated to take up the elute largely in water. Then, the elute was extracted with an equal volume of ethyl acetate five times and was dried down to dissolve with 70% ethanol.

HPLC

The sample extracted as described above was eluted through a Hichrom reverse-phase 25 -cm column packed with 5-μm particles of Spherisorb ODS-2 and analyzed with a Beckman Gold solvent delivery module controlled by a NEC PC800 computer and fitted with a Beckman L160 UV detector. Elution was performed at 3 ml/min with a gradient from 60% to 90% solvent B in solvent A (solvent A=2% acetic acid, solvent B=2% acetic acid in methanol) in 120 min. Eluted fractions were collected every minute, dried in a Speed Vac vacuum concentrator (Savant), and redissolved in 500 μl of 70% ethanol.

DIF assay

The activity of DIF for induction was measured as the ability to induce differentiation into stalk cells in isolated cells of strain V12M2 (Brookman *et al*., 1982). The cells were harvested from SM agar plates and washed free of bacteria. Then, 0.9×10^4 cells were plated in a tissue culture dish (ϕ=3 cm) under 1.5 ml of stalk salt solution (10 mM MES, 10 mM KCl, 2 mM NaCl, and 1 mM $CaCl_2$, pH 6.2)

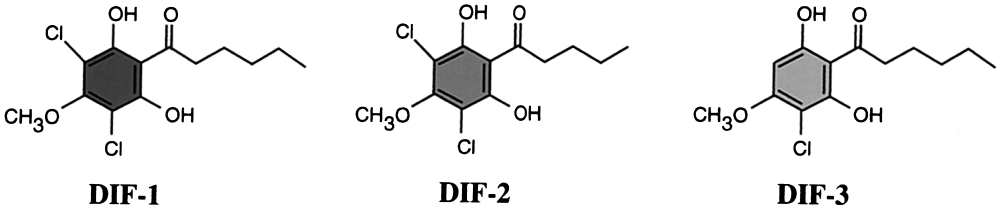

Fig 1. Structure of DIF-1, DIF-2 and DIF-3.

containing 5 mM cAMP and each HPLC fraction. Stalk cells were scored by phase contrast microscopy after 2 days of incubation at 22°C. One unit (U) of the activity induces 1% stalk cell differentiation in this assay.

RESULTS

Comparison of DRF-activities among DIF-1, DIF-2 and DIF-3

The chemical structures of DIF-1, DIF-2 and DIF-3 are shown in Fig. 1. DIF-1 and DIF-3 have a C_4 alkyl side chain, whereas DIF-2 has a C_3 alkyl side chain. DIF-3 is the monochlorinated analogue that is the initial degradation product of DIF-1 (Nayler *et al*., 1992). Distinct from DIF-1, DIF-2 and DIF-3 show minor biological activity for stalk cell induction. Here, we compared the DRF activity of DIF-1, DIF-2 and DIF-3 by placing *erkB*$^-$ cells on agar plates containing various concentrations of each DIF. To quantify the activity, the number of fruiting bodies formed after two days of incubation was counted (Fig 2). The DRF activity of DIF-1 was highest at 5 μM though it nearly reached this peak at 2 μM, but it was greatly reduced at 10 μM. At 10 μM, almost all the *erkB*$^-$ cells differentiated into stalk cells (data not shown). On the other hand, the activity of DIF-2 was hardly detectable until 2 μM, but increased at 5 and 10 μM in a dose-dependent manner. The number of fruiting bodies on DIF-2 agar at 5 μM was almost the same as that on DIF-1 agar at 1 μM and was about 25% of that of 5 μM DIF-1 agar. DIF-3 showed weak activity only at 10 μM. These results demonstrate that DIF-1 is most effective not only for stalk cell induction but also for the restoration of the development of *erkB*$^-$. The DRF activity of DIF-2 was 20~30% of that of DIF-1. The activity of DIF-3 was 15% of that of DIF-2 at 10 μM, that is, 3~4% of the activity of DIF-1. We could not see any striking difference among the phenotypes of *erkB*$^-$

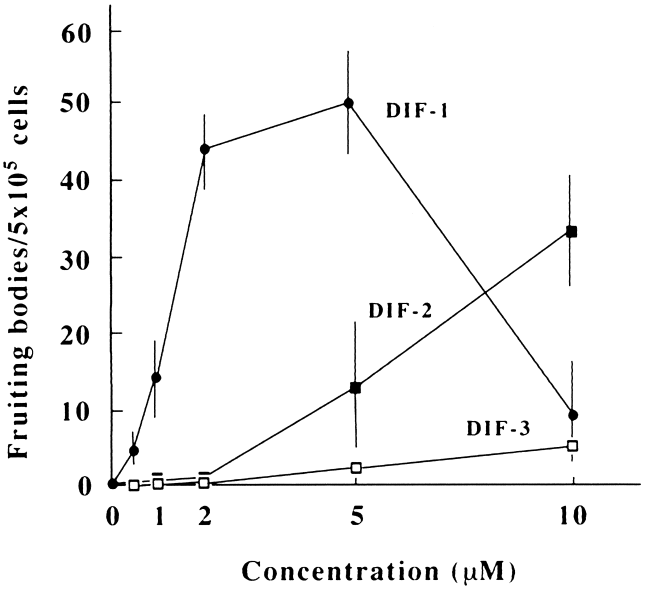

Fig 2. Comparison of DRF activity among DIF-1, DIF-2 and DIF-3. Aliquots (5 μl) of *erkB*$^-$ cell suspension were spotted on 1.6% hydrophobic agar plates containing DIF at 0.5, 1, 2, 5, or 10 μM. The number of fruiting bodies that formed on the plates was counted after two days of incubation.

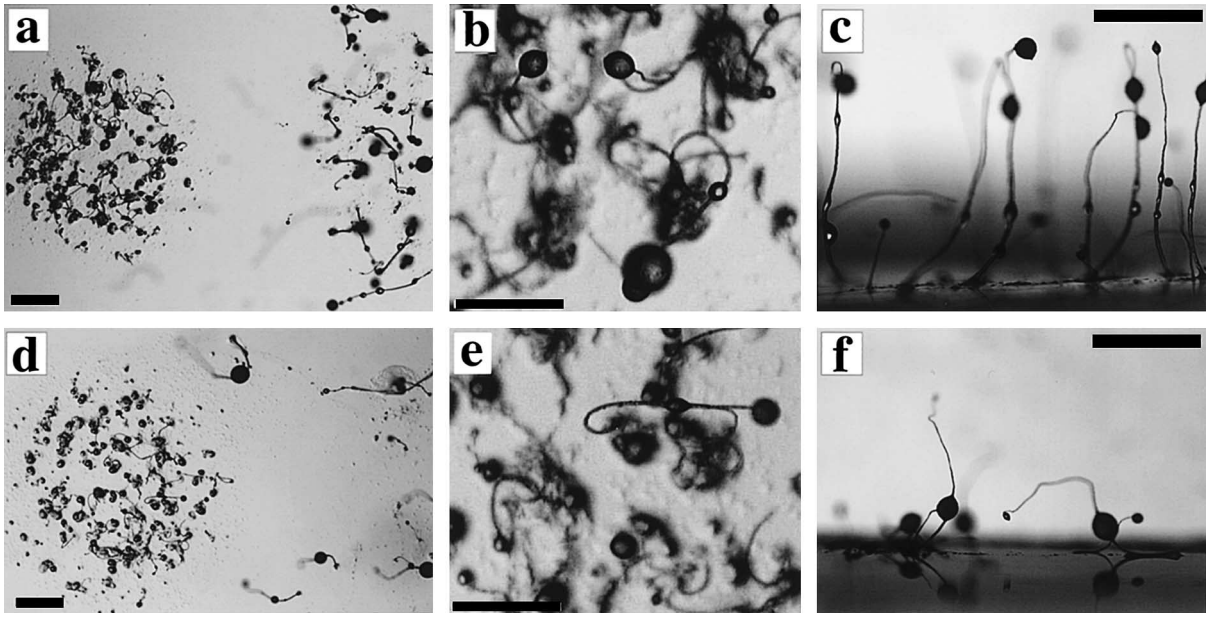

Fig. 3. Morphogenesis of *erkB*$^-$ cells rescued by DRFs secreted from Ax2 and *dmtA*$^-$. Effects of Ax2 (a) and *dmtA*$^-$ (d) on the morphogenesis of *erkB*$^-$ cells were examined by juxtaposing 5-μl aliquots of each cell suspension at the right of a spot of the *erkB*$^-$ cell suspension on 1.6% hydrophobic agar. (b) and (e) show a higher magnification of the spot of *erkB*$^-$ rescued by Ax2 (a) and *dmtA*$^-$ (d), respectively. Side view of the fruiting bodies formed from Ax2 (c) and *dmtA*$^-$ (f). The spore mass of a fruiting body formed from *dmtA*$^-$ often slipped toward the proximal region of a stalk (f).

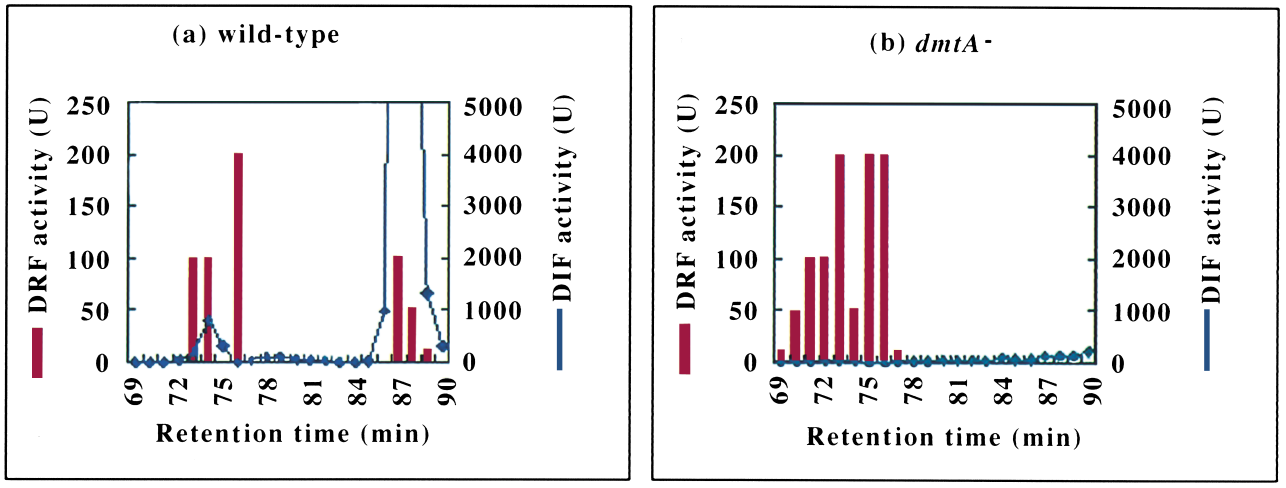

Fig. 4. DIF and DRF activities of HPLC fractions of the conditioned media prepared from the wild-type and DIF-less mutant *dmtA*$^-$. DIF and DRF activities contained in the conditioned media prepared from Ax2 (a) and *dmtA*$^-$ (b) were assayed after HPLC. DIF activity was measured as the ability to induce stalk cell differentiation in isolated cells of V12M2. One unit (U) of the activity represents 1% stalk cell differentiation. DRF activity was assayed by spotting a 0.5 μl aliquot of each fraction at the side of the spot of the *erkB*$^-$ cell suspension. DRF activity is represented as the highest magnitude of the dilution of each fraction which possesses DRF activity.

mutants restored by these DIFs (data not shown).

Restoration of the development of *erkB*$^-$ by DIF-less mutant *dmtA*$^-$

In an attempt to determine whether DIF-1 is the sole agent with strong DRF activity, we focused on the DIF-less mutant *dmtA*$^-$. The gene *dmtA* encodes the methyltransferase that catalyzes the final step in the pathway of DIF-1 biosynthesis. A *dmtA*$^-$ mutant was created by homologous recombination (Kay, 1998: Thompson and Kay, 2000). We tested whether the mutant is able to rescue the development of *erkB*$^-$ and revealed that the mutant secreted a DRF with activity similar to the wild-type (Fig 3). We also found that precursor molecules such as THPH (2,4,6-trihydroxyphenyl-1-hexan-1-one) and dichloro-THPH at the consecutive steps of DIF-1 biosynthesis never showed any DRF activity (data not shown). These indicate the existence of novel DRFs other than DIF-1 and its precursors.

Profile of DRF activity in conditioned media prepared from wild-type and *dmtA*$^-$ after HPLC

In order to identify novel DRFs, we pooled the conditioned media prepared from developing wild-type and *dmtA*$^-$ cells, respectively, and analyzed DRF activity after HPLC (see MATERIALS AND METHODS). First, the activity for stalk cell induction (DIF activity) in the wild-type preparation was assayed by the DIF assay. A single major DIF-peak was resolved in the fractions at 87–89 min (termed DIF-1 peak) where the authentic DIF-1 was eluted (Fig 4a). A minor DIF peak was also resolved in the fractions at 73–75 min (termed DIF-2 peak) at which the authentic DIF-2 was eluted (Fig 4a). DRF activity was also assayed in each HPLC fraction by the spot test. Two peaks of DRF activity were resolved in the wild-type preparation. One peak corresponded to the DIF-1 peak and the other nearly matched the DIF-2 peak. Thus, we speculated that the DRF activities in these two peaks resulted from DIF-1 and DIF-2. However, we obtained some unexpected results regarding the DRF peak around the DIF-2 peak. The DRF activity of DIF-2 was about 20~30% of that of DIF-1 as mentioned previously and the amount of DIF-2 was much less than that of DIF-1. Nevertheless, the DRF activity of the fractions at 73 and 74 min that should contain DIF-2 was as strong as that of the DIF-1 peak. Surprisingly, the fraction at 76 min demonstrated the strongest DRF activity (Fig 4a). As the fraction was supposed to lack any DIF (Fig 4a), these two DRF-peaks should be distinct from DIF-1 and DIF-2.

This prediction was supported by similar analysis of the conditioned media prepared from *dmtA*$^-$. Stalk-cell inducing activity by DIF-1 and DIF-2 was not detected at all in the mutant preparation, which is consistent with the report of Thompson and Kay (2000). Consistently, one of the major peaks of DRF activity in the DIF-1 fractions disappeared in the mutant preparation (Fig 4b). However, two other peaks of DRF-activity were left at around the DIF-2 peak of the wild-type (Fig 4b), indicating the existence of two novel DRFs other than DIF-1 and DIF-2. The development of the *erkB*$^-$ mutant rescued by the DRFs was phenotypically indistinguishable from that rescued by DIFs (data not shown).

DISCUSSION

We previously reported that diffusible factors released from developing wild-type cells rescue the development of the *erkB*$^-$ mutant and one of these factors is DIF-1 (Maeda and Kuwayama, 2000; Kuwayama *et al.*, 2000). In this study, we demonstrated that DIF-2 and DIF-3 also have

DRF activity. Much importantly, we revealed that at least two novel DRFs were secreted by the developing cells.

The bioactivity for stalk cell induction of DIF-2 is much weaker than that of DIF-1 and DIF-2 evokes the light scattering response in early developing *Dictyostelium* cells (Wurster and Kay, 1990). Therefore, we expected DIF-2 to possess particular functions *in vivo* related to DRF activity rather than stalk cell induction. This, however, is very unlikely since DIF-2 is less effective than DIF-1 in restoring the development of the *erkB*$^-$ mutant. At 5 μM, DIF-1 showed the highest level of DRF activity among the DIFs. DIF-2 and DIF-3 were respectively 20~30% and 3~4% as active as DIF-1, which is roughly comparable to the activity for stalk cell induction of DIF-2 and DIF-3 being 40% and 16% of that of DIF-1 (Masento *et al*., 1988). In this regard, it is notable that the DRF activity of DIF-1 was highest at 5 μM but declined to be less than that of DIF-2 at 10 μM. At high concentrations such as 10 μM, DIF-1 stimulated *erkB*$^-$ cells to differentiate into stalk cells without multicellular development. These results suggest that the DRF activity of DIF is related to the pathway of prestalk cell differentiation. As DIF-1 is known to increase intracellular proton and calcium levels (Inouye, 1988a; Schaap *et al*., 1996; Azhar *et al*., 1997), we expected such an increase to be involved in how DIF-1 rescues the development of *erkB*$^-$. Then, we tested whether a rise in the cytoplasmic calcium or proton concentration exerts a DRF-effect using calcium pump or proton ATPase inhibitors (Tsujioka *et al*., unpublished; Inouye, 1988b; Kubohara and Okamoto, 1994). Our preliminary results showed that these inhibitors induced stalk cell differentiation, but not the restoration of the mutant development (Tsujioka et al., unpublished data). However, we also found that 5 mM NH_4Cl eliminated the DRF activity of DIF-1 (Tsujioka *et al*., unpublished data), suggesting the involvement of a rise in the cytoplasmic proton concentration in the mechanism by which DIF-1 functions as a DRF.

Our study clearly showed the existence of at least two novel DRFs, though they remain to be identified. Both are diffusible in agar and have a similar hydrophobicity to DIF-2 as revealed by the fact that they eluted in nearly the same fractions as DIF-2 on HPLC. However, these DRFs never show stalk-inducing activity. Also, Maeda and Kuwayama (2000) have demonstrated that DRF activity becomes detectable after 8h of starvation and that the molecular masses of all DRFs are less than 3000. It is important to purify these DRFs and determine their structure. The analysis of these factors should provide insight into one more intercellular communication system of *Dictyostelium* during early development.

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