Distribution of gp138, a cell surface protein responsible for sexual cell fusion, among cellular slime moulds

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Sexual cell fusion occurs between NC4 and HM1, heterothallic strains of *Dictyostelium discoideum*. Several cell surface proteins relevant to the process have been identified. One of them, gp138, exists in fusion-competent cells of both NC4 and HM1, and is considered to be more concerned with membrane fusion than gamete recognition. In this study, we raised monoclonal antibodies against gp138 and examined gp138 distribution among strains and species of cellular slime moulds to confirm its importance in sexual cell fusion. All heterothallic and bisexual *D. discoideum* strains examined were found to possess gp138, while asexual and homothallic strains lacked it. The anti-gp138 monoclonal antibody detected several distinct proteins in homothallic strains and one in an asexual strain. Some of the former proteins appeared together with the increase in binucleated cells. Cells of *Dictyostelium mucoroides* and *Polysphondylium pallidum* did not possess proteins reactive to the monoclonal antibody. These results indicate that gp138 is common among, but restricted to, cross-matable strains of *D. discoideum*. Our results also support previously published molecular phylogenetic studies which suggest that homothallic and asexual strains of *D. discoideum* are remote from other strains of *D. discoideum* but are less distantly related to them than other species are.

Introduction

Sexual reproduction provides a means for shuffling genetic material. Without it, accumulation of advantageous mutations would be much slower (Kirkpatrick & Jenkins, 1989) and deleterious ones could not easily be eliminated (Kondrashov, 1988). Analysis of the origin and evolution of sexuality, therefore, is an interesting and important subject of basic biology, but its molecular basis is not well documented. Although molecular mechanisms of sexual cell fusion are being elucidated in several lower (Luporini & Heckmann, 1990; Herskowitz, 1989; Glabe *et al.*, 1991) and higher organisms (Sidhu & Guraya, 1991), very little phylogenetic analysis has been carried out.

The soil amoebae of the cellular slime moulds are unique and useful organisms for the study of sexual phenomena. They feed on bacteria and propagate by fission, while in due course they enter into the sexual cycle called macrocyst formation (Blaskovics & Raper, 1957; MacInnes & Francis, 1974). Entrance into the sexual cycle is controlled by environmental factors such as light and excess water (Nickerson & Raper, 1973; Erdos *et al.*, 1976). The mating system of cellular slime

moulds is polymorphic. Heterothallic, homothallic, bisexual and asexual strains have all been found in *Dictyostelium discoideum* (Erdos *et al.*, 1973; Robson & Williams, 1980) and alternate homothallism (Blaskovics & Raper, 1957) or heterothallism (Clark *et al.*, 1973; Erdos *et al.*, 1975; Francis, 1975) have been described in other species.

We have been studying the molecular aspects of sexual cell fusion in macrocyst formation using NC4 and HM1, complementary heterothallic strains in *D. discoideum*. Several cell surface components with possible relevance to the cell fusion between these strains have been identified (for a review, see Urushihara, 1992). One of these, gp138, has been shown to be a target antigen for cell-fusion-inhibiting antibodies (Suzuki & Yanagisawa, 1989*b*) and has been purified and characterized (Suzuki & Yanagisawa, 1990). This protein is detected in fusion-competent cells of both HM1 and NC4 and is therefore suspected to be responsible for membrane fusion and not for gamete recognition.

In this study, we raised monoclonal antibodies against gp138 and examined whether *D. discoideum* strains other than NC4 and HM1, and species other than *D. discoideum*, possessed gp138. It was widespread among heterothallic and bisexual strains in *D. discoideum*. Homothallic and asexual strains of *D. discoideum* lacked

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gp138 but retained proteins which shared the epitope to the antibody with gp138; other species did not.

Methods

Organisms and culture methods. NC4 (*mat A*) and HM1 (*mat a*, derived from V12) are heterothallic strains of *D. discoideum* and their mating properties have been described in detail (Suzuki & Yanagisawa, 1989a). WS472, WS583 and WS655 are *mat A* and WS7 and WS656 are *mat a* strains of *D. discoideum* (Erdos *et al.*, 1973; Robson & Williams, 1979), and were kindly provided by Dr K. Williams (Macquarie Univ., NSW, Australia). WS2162 and WS112B, bisexual strains which form macrocysts with either *mat A* or *mat a* strains (Robson & Williams, 1980), and WS584, an asexual strain without sexual potency (Erdos *et al.*, 1973), were gifts from Dr Welker's laboratory (Utah State Univ., USA). AC4 (Cavender & Raper, 1968) and ZA3A (Robson & Williams, 1980) are homothallic strains. These asexual and homothallic strains have been classified as being *D. discoideum* from morphological criteria, their fruiting bodies having discs at the bottom of the stalks, but recently this classification has been questioned (Briscoe *et al.*, 1987; Evans *et al.*, 1988) (see Discussion for details).

The Dm7 strain of *Dictyostelium mucoroides* (MacInnes & Francis, 1974) is homothallic and was provided by Dr Amagai (Tohoku Univ., Aoba, Sendai, Japan). A heterothallic strain of *Polysphondylium pallidum*, CK8 (Mizutani *et al.*, 1990), was isolated by Hagiwara. The names and mating properties of the strains used, which were confirmed at the beginning of the study, are given in Table 1. Each strain was maintained as fruiting bodies on nutrient SM-agar plates (Sussman, 1966) with *Klebsiella aerogenes* as a food source.

Preparation of fusion-competent cells. To obtain cells competent for sexual cell fusion (fusion-competent cells), growth-phase cells on SM-agar plates were cultured at 22 °C in a shaken liquid medium in darkness for 15 h unless otherwise stated. This cultured has been shown to render NC4 and HM1 cells capable of immediate cell fusion (Saga *et al.*, 1983).

Partial purification of gp138. gp138 was partially purified using WGA affinity columns (Suzuki & Yanagisawa, 1990). Cell ghosts (crude membrane fraction) (Sussman & Boschwitz, 1975) were prepared from fusion-competent NC4 cells and solubilized in a Tris buffer (10 mM-Tris/HCl, pH 7.4) containing 0.5% cholic acid and 0.5% deoxycholic acid and centrifuged at 14000 r.p.m. for 10 min. The supernatant was recentrifuged for clarification and loaded onto a column of WGA-agarose equilibrated with the Tris buffer containing 0.15% deoxycholic acid (DOC-Tris). After extensive washing with DOC-Tris, the bound fraction was eluted with DOC-Tris containing 0.2 M-N-acetyl-D-glucosamine.

Raising and screening hybridomas. Partially purified gp138 was intraperitoneally injected into BALB/c mice together with aluminium hydroxide as adjuvant. After three weeks, the same quantity of gp138 was injected into their eye sockets. Three days later, the mice splenocytes were fused with P3U1 (P3X63Ag8-U1) myeloma cells by the conventional hybridoma technique. The resulting hybridomas were first screened for production of antibodies that bound to partially purified gp138 by the enzyme-linked immunosorbent assay. The selected hybridomas were further screened for specificity to gp138 by immunoblotting. Positive hybridomas were then cloned by plating at the limiting dilution.

Gel electrophoresis and immunoblotting. Slab SDS-PAGE was performed by the method of Laemmli (1970) with minor modifications. For immunoblotting, proteins in a gel were transferred to a nitrocellulose membrane (0.45 µm, Schleicher & Schuell), which was then incubated with antibodies for 1 h at room temperature. The membrane was further incubated with peroxidase-conjugated anti-

mouse IgG (Jackson) or protein A (EY Laboratories) for another hour and then developed with 0.03% diaminobenzidine.

Nuclear staining. To estimate the extent of cell fusion in the culture of homothallic strains, aliquots of cell suspension were taken for nuclear staining with Hoechst 33258 by the method described previously (Urushihara *et al.*, 1990). Numbers of cells with 1, 2, 3 and more nuclei were counted and the percentage of nuclei in multi-nucleated cells to the total number of nuclei was calculated and used as the index of cell fusion. Duplicates of more than 200 cells were examined for each sample.

Results

Specificity of the anti-gp138 monoclonal antibody

Five monoclonal antibodies specific to gp138 were obtained. One of them, EF11, was used for this study. It gave a single band in immunoblots of both total proteins of fusion-competent NC4 cells and of partially purified gp138 (Fig. 1). This band comigrated with gp138 detected by FRA-6, a rabbit antiserum raised against purified gp138 (Suzuki & Yanagisawa, 1990). Specificity of EF11 to gp138 was confirmed also by two-dimensional gel electrophoresis by the methods of Hirabayashi (1981) (data not shown).

Detection of gp138 in heterothallic strains of *D. discoideum*

Various heterothallic strains of *D. discoideum* were examined for expression of gp138 after cultivation in a liquid medium for 15 h in darkness (Fig. 2). EF11

Fig. 1. Specificity of EF11, an anti-gp138 monoclonal antibody. Whole proteins of fusion-competent NC4 cells (a and b) and partially purified gp138 (c and d) were subjected to SDS-PAGE and immunostained either with FRA-6 antiserum (a and c) or with EF11 (b and d). The arrow indicates the position of gp138.

Fig. 2. Detection of gp138 in heterothallic strains of *D. discoideum*. Whole proteins obtained from 1×10^5 fusion-competent cells of (a) NC4, (b) WS472, (c) WS583, (d) WS655, (e) HM1, (f) WS7, and (g) WS656 were subjected to SDS-PAGE, transferred to nitrocellulose membrane and immunostained with EF11. Lanes a–d are *mat A*, and e–g *mat a* strains. The arrow indicates the position of gp138.

detected gp138 in HM1. This is in accord with the previous finding that, although gp138 was first identified in NC4, it exists also in HM1 (Suzuki & Yanagisawa, 1989b). Furthermore, all other heterothallic strains in *D. discoideum* examined also possessed gp138. These included both *mat A* (WS472, WS583 and WS655) and *mat a* strains (WS7 and WS656). An additional band of about 95 kDa is seen in Fig. 2 but it appeared without reproducibility. This band seems to be due to background staining. Except for this, gp138 was the only protein that reacted with EF11 in all the heterothallic strains shown in Fig. 2.

Reactivity of anti-gp138 antibody with other strains of *D. discoideum*

We extended the examination to nonheterothallic strains, that is, bisexual, homothallic and asexual strains which had been morphologically assigned to *D. discoideum*. As can be seen in Fig. 3, a bisexual strain, WS2162, possessed gp138 as the only band detected by EF11.

Fig. 3. Detection of gp138 in atypical strains of *D. discoideum* and in other species. Cells were cultured in a shaken liquid medium to render them fusion-competent as described in Methods. Whole proteins obtained from 1×10^5 cells of (a) NC4, (b) WS2162, (c) WS112B, (d) AC4, (e) ZA3A, (f) WS584, (g) Dm7 and (h) CK8 were subjected to SDS-PAGE and either transferred to nitrocellulose membrane for immunostaining with EF11 (A) or directly stained with CBBR (B). The arrow indicates the position of gp138.

Fig. 4. Time course of multinucleated-cell formation (a) and appearance of EF11-reactive proteins (b) in a liquid culture of AC4 cells. Cells on SM-agar plates were cultured in a liquid medium at 22 °C in darkness. At the times indicated, cells were taken for nuclear staining and for SDS-PAGE followed by immunostaining with EF11. In (a) duplicate samples were examined and their means were plotted. Standard deviations are shown by bars. The arrow in (b) indicates a band appearing in parallel with formation of multinucleated cells.

Another bisexual strain, WS112B, gave similar results except that the band of gp138 tended to be somewhat broader. This tendency was also observed in HM1 (for example, lane *e* in Fig. 2) but not in NC4 or WS2162.

In contrast to the strains mentioned above, homothallic and asexual strains gave entirely different results. EF11 appeared to react with proteins of distinct molecular mass from gp138 in the immunoblot of homothallic strains, AC4 and ZA3A, and an asexual strain, WS584. AC4 possessed two EF11-reactive proteins of 190 and 96 kDa. ZA3A possessed a 66 kDa protein with enormous reactivity to EF11 and several proteins of 195, 57, 52, and 46 kDa with much weaker reactivity, while WS584 possessed a single EF11-reactive protein of 185 kDa.

Those EF11-reactive bands in AC4 and ZA3A may be related to sexual cell fusion. Alternatively, they might simply share the epitope to EF11 with gp138, having

Fig. 5. Time course of multinucleated-cell formation (a) and appearance of EF11-reactive proteins (b) in a liquid culture of ZA3A cells. Cells on SM-agar plates were cultured in a liquid medium at 22 °C in darkness. At the times indicated, cells were taken for nuclear staining and for SDS-PAGE followed by immunostaining with EF11. In (a) duplicate samples were examined and their means were plotted. Standard deviations are shown by bars. Arrows in (b) indicate bands appearing in parallel with formation of multinucleated cells.

nothing to do with cell fusion. In order to clarify this point, occurrence of cell fusion and expression of EF11-reactive proteins in these strains were examined in parallel. To do this, growth-phase cells on SM-agar plates were cultured in a liquid medium in darkness, and at appropriate times, a small number of cells were taken from the culture and processed for nuclear staining to detect multinucleated cells and for electrophoresis to detect EF11-reactive proteins.

As shown in Fig. 4(a) the multinucleated cells in AC4 were barely above 1 % at time 0; these probably represent cells in mitosis. The percentage of multinucleated cells gradually increased with time and reached a plateau at around 16 h. In the immunoblotting pattern of AC4 (Fig. 4b), the 96 kDa band was absent at time 0,

Table 1. Detection of proteins reactive to the anti-gp138 monoclonal antibody

Species*	Strain	Mating property	EF11-reactive protein†	
			gp138	Other‡
<i>D. discoideum</i>	NC4	Heterothallic (<i>mat A</i>)	+	—
	WS472	Heterothallic (<i>mat A</i>)	+	—
	WS583	Heterothallic (<i>mat A</i>)	+	—
	WS655	Heterothallic (<i>mat A</i>)	+	—
	HM1	Heterothallic (<i>mat a</i>)	+	—
	WS7	Heterothallic (<i>mat a</i>)	+	—
	WS656	Heterothallic (<i>mat a</i>)	+	—
	WS2162	Bisexual	+	—
	WS112B	Bisexual	+	—
	AC4	Homothallic	—	+
	ZA3A	Homothallic	—	+
<i>D. mucoroides</i>	WS584	Asexual	—	+
	Dm7	Homothallic	—	—
<i>P. pallidum</i>	CK8	Heterothallic	—	—

* Assignment of homothallic and asexual strains to *D. discoideum* does not seem to be appropriate (see text) but morphological classification is adopted in this instance.

† + and — indicate the presence and absence of EF11-reactive proteins, respectively.

‡ Molecular masses of the reactive proteins were, 190 and 96 kDa in AC4, 195, 66, 57, 52 and 46 kDa in ZA3A, and 185 kDa in WS584.

appeared from 9 h of cultivation and was most intense at 16 h, while the 190 kDa band was detected at 0 h and its intensity remained fairly constant throughout the culture period. Very similar parallelism was observed in ZA3A. At time 0, only the 195 kDa band was present and the major 66 kDa band and less intense bands below it appeared at 9 h (or 15 h for faint bands), and became most intense at 15 h (Fig. 5b) in correspondence with the increase of multinucleated cells (Fig. 5a). The faint bands below the 66 kDa band might be its degradation products, but this is still uncertain. Thus, expression of the 96 kDa protein in AC4 and the 66 kDa protein and the lower-molecular-mass bands in ZA3A apparently correlated with the formation of multinucleated cells in those strains. These proteins may be related to sexual cell fusion of the relevant strains. gp138 was not detected at any time of the culture period in either one of the homothallic strains. Since the asexual strain did not undergo sexual cell fusion to produce multinucleated cells, it was not possible to assess the relationship of the 185 kDa band in WS584 to sexual cell fusion.

Absence of EF11-reactive proteins in species other than *D. discoideum*

Strains of different species from *D. discoideum*, Dm7 in *Dictyostelium mucoroides* and CK8 in *Polysphondylium pallidum*, were examined. Fusion-competent cells of Dm7

and CK8 were obtained by culturing cells in liquid for 18 h and 17 h, respectively, and were subjected to SDS-PAGE. When the electrophoresis gel was stained with Coomassie Blue, comparable amounts of proteins to *D. discoideum* strains were seen in both of the species (Fig. 3b). However, EF11 did not detect any proteins in these strains (Fig. 3a). Alternative methods to obtain fusion-competent Dm7 cells by moderate starvation (Filosa & Dengler, 1972) or cold treatment (Suzuki *et al.*, 1992) were attempted. In neither case were EF-11 reactive proteins detected. Thus, it was concluded that *D. mucoroides* and *P. pallidum* did not possess gp138, nor proteins sharing the epitope to EF11 with gp138. All the above results of immunostaining are summarized in Table 1. Obvious discreteness is seen between heterothallic and bisexual (typical) strains and homothallic and asexual (atypical) strains of *D. discoideum* and between *D. discoideum* and the other two species.

Discussion

The cell surface glycoprotein gp138 was previously shown to be responsible for sexual cell fusion between NC4 and HM1, complementary heterothallic strains in *D. discoideum* (Suzuki & Yanagisawa, 1989b). The purpose of the present study was primarily to investigate the universality of this protein in order to confirm its importance, and secondly, to determine its relationship to the divergency of mating systems in cellular slime moulds. Our results demonstrated the presence of gp138 as a single protein reactive to EF11, an anti-gp138 monoclonal antibody, in all heterothallic and bisexual strains of *D. discoideum* examined. However, homothallic and asexual strains which had been assigned to *D. discoideum* from morphological criteria did not possess gp138 but were found to contain distinct proteins recognized by EF11. Cell lysates obtained from species other than *D. discoideum*, i.e. *D. mucoroides* and *P. pallidum* contained no EF11-reactive proteins. All these results indicate that gp138 is common among but restricted to cross-matable strains of *D. discoideum* and suggest that molecular mechanisms of sexual cell fusion are variable among cellular slime moulds.

Several recent studies on polymorphisms of protein size (Briscoe *et al.*, 1987) and of restriction fragment length (Evans *et al.*, 1988) suggest that classification of cellular slime moulds based on morphology of fruiting bodies is not appropriate for certain strains. The present results are in striking agreement with these molecular phylogenetic arguments, providing further supportive evidence for each of their claims as follows. Firstly, they reported that homothallic and asexual strains which had been morphologically assigned to *D. discoideum* showed different patterns of polymorphism from heterothallic

and bisexual strains of *D. discoideum*, and seemed to be remote from typical (heterothallic and bisexual) *D. discoideum* strains. In our study, the former atypical *D. discoideum* strains did not possess gp138 and the latter typical ones possessed gp138. Secondly, however, their results indicated that the homothallic and asexual strains were less different from typical *D. discoideum* strains than were morphologically distinct species such as *D. mucoroides* and *P. pallidum*. The present results showed that the former possessed EF11-reactive proteins, while the latter did not possess any EF11-reactive proteins. In this respect, it may be worth noting that we previously reported that the cell-fusion promoting factor was shared in heterothallic and homothallic systems and suggested that their molecular mechanisms for sexual cell fusion might be similar (Urushihara *et al.*, 1990). Finally, they claimed that, since two homothallic strains, AC4 and ZA3A, were different in protein and nucleic acid polymorphisms and not closely related, they should be assigned to separate species. We further demonstrated that patterns of antigenic proteins reactive to EF11, including the molecular entities correlated with formation of multinucleated cells, were different between AC4 and ZA3A.

One report suggests the possibility that homothallic strains undergo sexual cell fusion with heterothallic ones in *D. discoideum*. Robson & Williams (1980) obtained macrocysts in the co-culture of homothallic and heterothallic strains. Although their results indicate that homothallic strains can form macrocysts in the presence of heterothallic ones, this does not necessarily mean that sexual cell fusion occurred between those strains. It is also uncertain whether two homothallic strains mutually fuse to produce macrocysts or not. To clarify these points, genetic analyses should be carried out. Because of poor germination of macrocysts, this kind of study has not yet been done, but should ultimately be carried out.

The fact that homothallic and asexual strains did not possess gp138 and that *D. mucoroides* and *P. pallidum* did not possess any EF11-reactive proteins indicates dissimilarity in mechanisms of sexual cell fusion among those strains. However, it is premature at this stage of research to judge whether or not this is the reason for the absence of cross-mating with typical *D. discoideum* strains. Conversely, the polymorphism of EF11-reactive proteins might itself be the result of the lack of cross-mating, as simple accumulation of spontaneously-occurring independent changes would be enough to create protein polymorphisms among species without genetic exchange. Further analysis of molecular mechanisms of sexual cell fusion will elucidate these points.

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