Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 6596–6600, November 1983
Developmental Biology

Involvement of a cell-surface glycoprotein in the cell-sorting process of *Dictyostelium discoideum*

(sorting assay/prespore and prestalk cells/glycoprotein gp150/Fab inhibition)

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Communicated by Hewson Swift, July 25, 1983

ABSTRACT **Cell sorting among prespore cells and prestalk cells in *Dictyostelium discoideum* was studied by using fluorochrome-labeled cells in an *in vitro* assay. Labeled prestalk cells first formed randomly mixed aggregates with unlabeled prespore cells. Then cells began to sort out from each other. About 3–4 hr later, prestalk cells became clustered at one pole of the aggregate. Aggregates deposited on an agar surface underwent morphogenesis and formed migrating slugs within 3 hr. The addition of Fab fragments directed against a cell-surface glycoprotein of M_r 150,000 (gp150) to the cell mixture completely inhibited the cell-sorting phenomenon. Morphogenesis of such aggregates on agar was also delayed by 5 hr. However, inclusion of Fab fragments directed against the endogenous lectins, the contact site A glycoprotein, or vegetative cells had no detectable effect on cell sorting or morphogenesis of these reconstituted aggregates.**

The cellular slime mold *Dictyostelium discoideum* is a popular model for the study of cell–cell interaction. Vegetative cells contain EDTA-sensitive cell-binding sites, which mediate the formation of loose cell aggregates in liquid cultures. These aggregates are completely dissociated into single cells in 1 or 2 mM EDTA (1). During development, cells form EDTA-resistant binding sites on their surfaces at the aggregation stage (2). Cells form tight aggregates, which are resistant to EDTA dissociation up to a concentration of 15 mM. The endogenous lectins discoidin I and II (3, 4) and a contact site A glycoprotein of M_r 80,000 (gp80) (5, 6) have been implicated in mediating cell–cell adhesion at the aggregation stage. These stable aggregates give rise to pseudoplasmodia or slugs. At the slug stage, cells are organized in a spatial pattern in which the posterior prespore zone and the anterior prestalk region are clearly distinguishable by various morphological and biochemical criteria (7).

Cell sorting probably takes place at an early postaggregation stage, leading to pattern formation in the slug (8). Evidence for the sorting out of cells at the slug stage was demonstrated by Bonner (9) in 1959. Subsequently, Takeuchi (10) showed that disaggregated anterior cells labeled with [^{3}H]thymidine, after mixing randomly with unlabeled disaggregated posterior cells, were able to reoccupy the anterior position of the new slugs. Similar experiments have confirmed these observations by using different labeling techniques (11, 12).

Recently, a surface glycoprotein of M_r 150,000 (gp150) has been implicated in mediating cell cohesion (13, 14). We have demonstrated that univalent antibodies directed against gp150 inhibit specifically the reassociation of cells in postaggregation stages (15). In the present report, we describe a simple and reproducible *in vitro* assay for cell sorting. We also show that Fab directed against gp150 can block cell sorting, whereas Fab fragments directed against gp80 or discoidin have no adverse effect on this phenomenon.

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MATERIAL AND METHODS

Cell Strain and Culture Conditions. *D. discoideum* strain NC4 was used in all experiments. Cells were grown on agar plates with *Klebsiella aerogenes* and deposited for synchronized development on Whatman filters according to Sussman (16).

Labeling of Cells with Fluorochrome. Fluorochrome was conjugated to cell-surface components according to the method of Springer and Barondes (17) with slight modification. NC4 cells at early culmination stage (17–18 hr) were dissociated in 17 mM Na_2/K phosphate buffer (pH 7.5) containing 20 mM EDTA. Tetramethylrhodamine isothiocyanate (TRITC) or fluorescein isothiocyanate (FITC) was prepared just before use at 25–30 mg/ml in dimethyl sulfoxide. To each milliliter of cell suspension (3×10^7 cells per ml), 10 μl of dye solution was added and conjugation was carried out at 20°C for 5 min. Cells were washed and then resuspended for cell separation. Cell viability was checked by trypan blue exclusion; >98% of cells were viable at this step.

Separation of Prespore and Prestalk Cells. The renografin gradient centrifugation procedure described by us previously was followed (15).

Cell-Cohesion Assay. Intercellular cohesiveness was quantitated by the method of Gerisch (1) with slight modifications (18).

Cell-Sorting Assay. TRITC- or FITC-labeled prestalk cells were mixed with unlabeled prespore cells at a ratio of 1:2 in Bonner's salt solution containing 10 mM NaCl, 10 mM KCl, and 3 mM $CaCl_2$. Cells were resuspended gently at $3–5 \times 10^6$ cells per ml and mixed well. The cell mixture was rotated at 60 rpm and aggregates were placed in a shallow trough on a glass slide and observed under a Leitz Orthoplan fluorescence microscope. To follow the morphogenesis of these aggregates, cell clumps were deposited on a plain 2% agar plate 1 hr after rotation in liquid medium. Plates were placed in the dark and scored at regular intervals for the number of migrating slugs and fruiting bodies.

Preparation of Fab Fragments. Rabbit antiserum directed against gp150 was obtained from J. Geltosky (Scripps Clinic and Research Foundtion). Details of characterization of this antiserum have been reported by Geltosky *et al.* (14). Monovalent Fab fragments were prepared by papain digestion of purified IgG (19). Fab fragments were also prepared from antisera raised against the endogenous lectins discoidin I and II (20) and the contact site A glycoprotein gp80.

Quantitation of Fab Binding. Cells (10^6) were mixed with Fab for 60 min at 4°C and the unbound Fab was removed by

Abbreviations: FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate.

repeated washing. A FITC-conjugated second antibody (sheep IgG) against rabbit IgG was added and incubated for 30 min. Cells were washed and lysed in 0.1 M NaOH. Fluorescence was measured in a Perkin-Elmer fluorometer by using an excitation wavelength of 490 nm and an emission wavelength of 530 nm.

RESULTS

Cell Sorting Among Prestalk and Prespore Cells. Prespore cells and prestalk cells were separated on a renografin density gradient as described (15). Proteolytic enzymes were not used to dissociate cells and they were able to recover quickly. TRITC or FITC was conjugated to cell-surface components prior to cell separation. Under our conditions, all cells were labeled fairly uniformly and the fluorescence was stable for a prolonged period of time. Fluorochrome conjugation did not affect either the EDTA-sensitive or EDTA-resistant type of cell–cell binding. These cells were able to undergo normal morphogenesis when deposited on an agar plate. After FITC-labeled prespore cells were mixed with TRITC-labeled prestalk cells for 5 hr, <5% of cells was found to contain both types of fluorescence, indicating no significant exchange of fluorochrome.

To assay for cell sorting, FITC-labeled prestalk cells were randomly mixed with unlabeled prespore cells and aggregates were observed under a fluorescence microscope at regular intervals. As shown in Fig. 1, the fluorescent cells adhered randomly with the unlabeled cells and fairly large aggregates were formed within the first 60 min. During the next 60 min, cells of the same type began to associate with one another, giving rise to clusters of fluorescent cells and unlabeled cells. At the same time, the fluorescent cells appeared to occupy the peripheral region of the aggregate, leaving the unlabeled cells in the middle. By 2–3 hr, most of the fluorescent prestalk cells were forming the outer layer of the aggregate, wrapping around an inner core of unlabeled prespore cells. Between 3 and 4 hr, the prestalk cells on the outer periphery began to move to one pole of the aggregate. The formation of polarity in the aggregate was similar to the pattern observed at the tip stage during early slug development. Indeed, when aggregates at this stage were removed from the liquid medium and deposited on a solid agar surface, they showed tip formation within 30–45 min.

In Fig. 1, micrographs were taken with a focal plane across the middle of the aggregate and fluorescent cells on the upper periphery were not readily observable. Therefore, we gently flattened aggregates from the 3-hr sorting stage under a coverslip to allow better visualization of cells on the outer periphery. Patterns typical of those observed are shown in Fig. 2. About 75% of the outer surface of such an aggregate was covered with TRITC-labeled prestalk cells. (See Fig. 4*d*, in which the sorting pattern shows that the aggregate was in the process of polarity formation.)

Inhibition of Cell Sorting by Fab Against gp150. We previously found that a major concanavalin A binding protein gp150 accumulated on the cell surface between 6 and 15 hr of development (13). This glycoprotein was subsequently purified and used to raise specific antibodies in rabbit (14). Fab fragments derived from the antiserum were effective in blocking the EDTA-resistant cell–cell binding among cells in postaggregation stages but not among aggregation-stage cells (15). If, indeed, gp150 were involved in the cell-sorting process, Fab against gp150 should interfere with the cell-sorting process in our *in vitro* assay. To test this, it was necessary to demonstrate that Fab against gp150 had no inhibitory effect on the EDTA-sensitive binding sites, which would enable the cells to form large aggregates, even though their EDTA-resistant binding sites had been blocked by anti-gp150 Fab fragments. Fig. 3 shows that reaggregation of culmination-stage cells was completely blocked in the presence of EDTA and Fab against gp150, whereas Fab or EDTA alone had very little effect on the kinetics of cell reassociation.

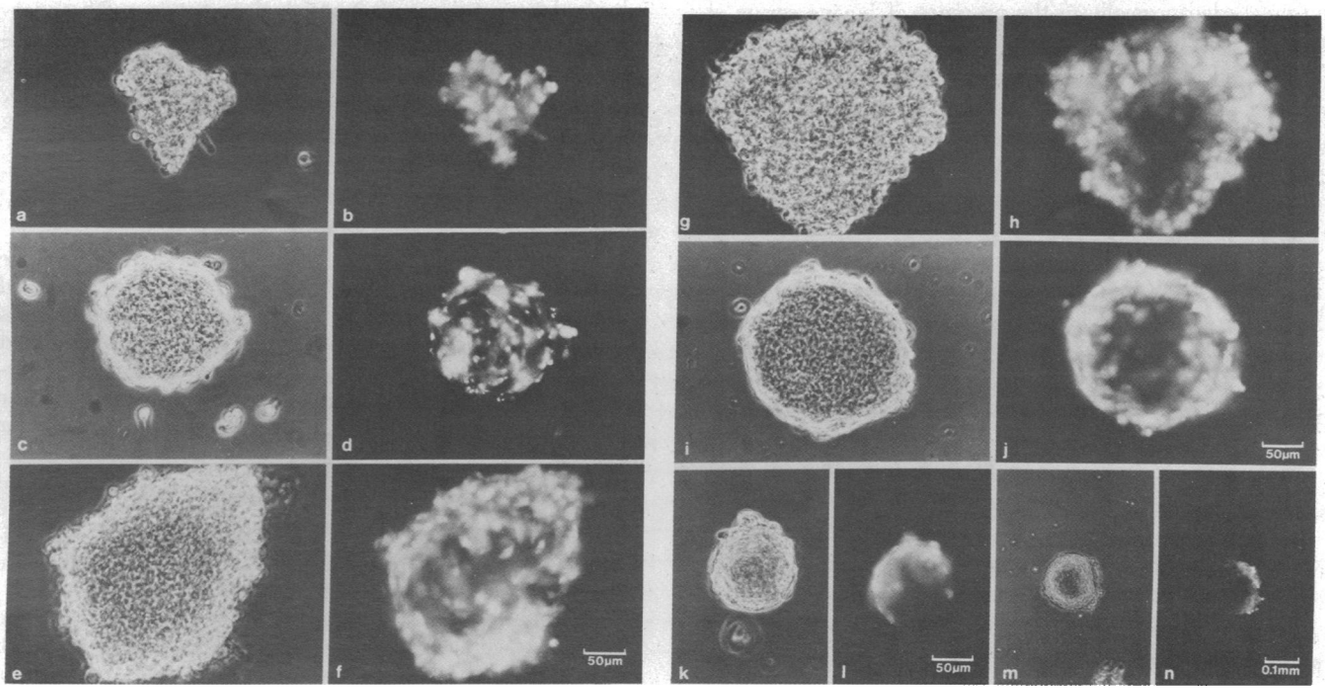

FIG. 1. Time course of the cell-sorting process. FITC-labeled prestalk cells were mixed with unlabeled prespore cells at a ratio of 1:2 and allowed to reaggregate in Bonner's salt solution. Aggregates were placed in a shallow trough and observed under the fluorescence microscope at intervals. Pairs of phase and fluorescence micrographs of aggregates at different stages of sorting are shown. (*a* and *b*) Phase-contrast and fluorescence micrographs of an aggregate at 30 min after mixing; (*c* and *d*) 1.5 hr; (*e* and *f*) 2 hr; (*g* and *h*) 2. 5 hr; (*i* and *j*) 3 hr; (*k* and *l*) 3.5 hr; and (*m* and *n*) 4 hr.

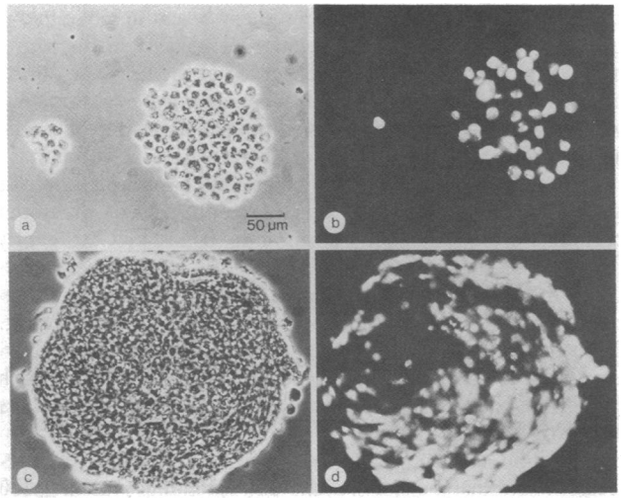

FIG. 2. Cell sorting among prespore and prestalk cells. TRITC-labeled prestalk cells were mixed with unlabeled prespore cells and aggregates were gently flattened under a coverslip. (*a* and *b*) Phase-contrast and fluorescence micrographs of an aggregate at 30 min after mixing; (*c* and *d*) a 3-hr aggregate.

When Fab against gp150 was added to prespore and prestalk cells before aggregate formation, cell sorting among the two cell types was completely blocked (Fig. 4 *a* and *b*). To rule out the possibility that the Fab inhibited cell locomotion and not sorting, cells rotated in the presence of Fab for 3 hr were dissociated and allowed to attach to glass for microscopic observation. These cells were able to undergo normal amoeboid movement in the presence of Fab. More than 95% of these cells were viable by the trypan blue exclusion assay. Therefore, it is evident that anti-gp150 Fab did not inhibit cell locomotion and it had no toxic effect on cells.

Contrary to results obtained with anti-gp150 Fab, cell sorting proceeded normally in the presence of bovine serum albumin or Fab prepared from preimmune rabbit serum (Fig. 4 *c* and *d*). Also, cells were reassociated in the presence of Fab

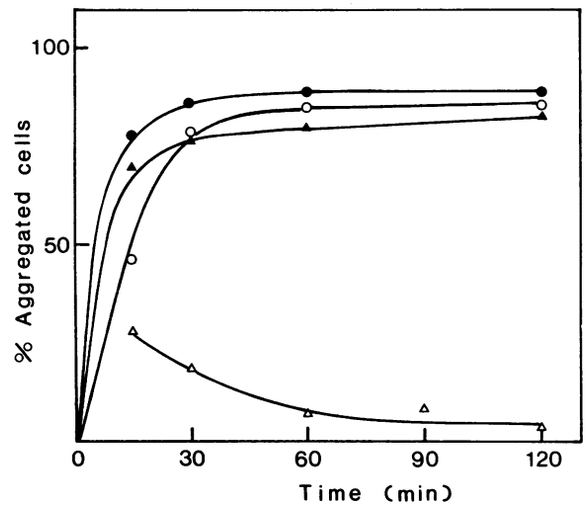

FIG. 3. Effect of Fab fragments against gp150 on cell reassociation. Prestalk and prespore cells were separated by gradient centrifugation and then mixed at a ratio of 1:2. The kinetics of cell reassociation were monitored under different conditions. (△) Cells reaggregated in the presence of 0.5 mg of Fab per ml against gp150 and 5 mM EDTA; (▲) cells aggregated in 5 mM EDTA; (○) cells reaggregated in the presence of 0.5 mg of Fab per ml without EDTA; and (●) control cells reaggregated in the absence of both EDTA and Fab.

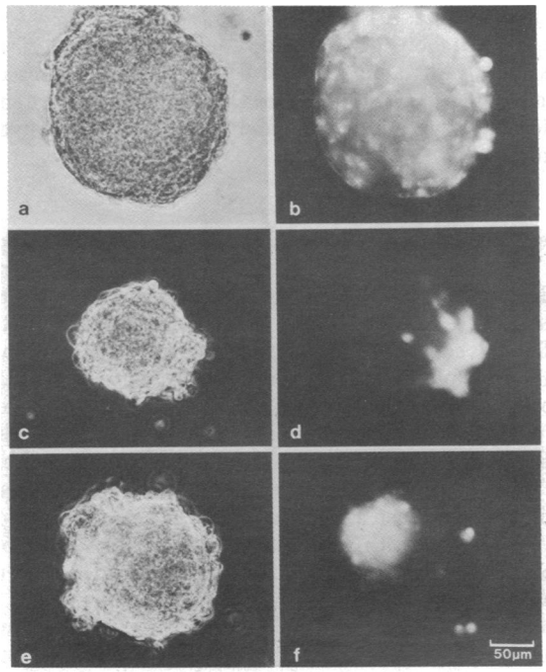

FIG. 4. Inhibition of cell sorting by Fab directed against gp150. Cell sorting was assayed as described in the legend to Fig. 1. Right after the two cell populations were mixed, Fab against gp150 or against other cell-surface components was added at a final concentration of 1 mg/ml in each case. Pairs of phase-contrast and fluorescence micrographs were taken 5 hr after the initial mixing of cells. (*a* and *b*) Inhibition of cell sorting in the presence of Fab against gp150; (*c* and *d*) cell sorting in the presence of Fab derived from preimmune rabbit serum; (*e* and *f*) cell sorting in the presence of Fab against discoidin I and II. Results obtained by using anti-gp80 Fab or antivegetative cell Fab were similar to *e* and *f*.

directed against the contact site A glycoprotein gp80, endogenous lectins discoidin I and II, or vegetative cell. Under similar conditions, the amount of anti-gp150 Fab bound to cells was slightly higher than anti-gp80 Fab or antidiscoidin Fab, whereas antivegetative cell Fab showed a 2-fold higher binding (Table 1). In all cases, except when Fab against gp150 was added, the fluorescent prestalk cells sorted out from the prespore cells and clear polar organization of the two cell types was observed within a period of 3–4 hr (Fig. 4 *e* and *f*).

Effect of Anti-gp150 Fab on Morphogenesis. To follow the pattern of cell sorting during morphogenesis, we transferred the reconstituted aggregates containing labeled prestalk cells and unlabeled prespore cells after 1 hr of rotation onto a solid agar surface and determined the time required for slug formation. About 90% of the aggregates were in slug form 3–4 hr after plating on agar (Table 1). Many continued to migrate for a period of 6–10 hr before culminating in the formation of fruiting bodies. Most of the labeled cells were localized in the anterior zone of the migrating slug and a clear demarcation line between the anterior zone and the posterior region was evident. Less than 10% of the FITC-labeled prestalk cells were estimated to remain behind in the posterior region.

The effect of Fab against gp150 on morphogenesis was examined on aggregates deposited on solid agar for development. After 6 hr, most of the aggregates formed in the presence of anti-gp150 Fab remained as round mounds on the agar surface (Fig. 5*a*), whereas all of the control aggregates had already developed into migrating slugs. In contrast, aggregates formed in the presence of Fab against discoidin, gp80, or vegetative cell underwent normal morphogenesis with a time frame similar to

Table 1. Inhibitory effect of Fab against gp150 on morphogenesis of reconstituted aggregates

Fab type	Relative binding of Fab*	Time after cell mixing, hr	% aggregates in various morphological forms†		
			Round mound	Slug	Fruiting body
Control (−Fab)	5	5	7	93	0
		10	7	85	8
Anti-gp150 Fab	45	5	78	22	0
		10	36	64	0
Anti-gp80 Fab	37	5	10	90	0
		10	10	90	0
Antivegetative cell Fab	81	5	11	89	0
		10	8	86	6
Antidiscoidin I and II Fab	30	5	8	92	0
		10	8	90	2

* Relative amounts of Fab bound per 10^6 cells were estimated by binding of a FITC-conjugated second antibody. Values represent relative fluorescence expressed in arbitrary unit.

† Reaggregation of cells was carried out in the presence of different types of Fab (1 mg/ml) for 60 min before the reassociated aggregates were transferred to 2% agar plates. Results represent the average of two independent experiments.

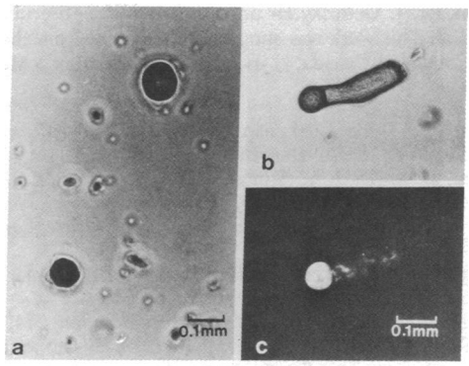

FIG. 5. Inhibition of morphogenesis in reconstituted aggregates. FITC-labeled prestalk cells were mixed with unlabeled prespore cells in the presence of either Fab against gp150 or Fab against discoidin I and II in liquid medium. After 1 hr of rotation, aggregates were deposited on a plain agar surface and micrographs were taken 6 hr later. (*a*) Phase-contrast micrograph of aggregates previously exposed to Fab against gp150; (*b* and *c*) phase-contrast and fluorescence micrographs of a migrating slug developed from an aggregate reassociated in the presence of Fab against discoidin I and II. Control showed results similar to *b* and *c*.

that of the control (Fig. 5 *b* and *c*).

Results were also quantitated by estimating the percentage of aggregates in slug form at different times (Table 1). More than 90% of control aggregates and aggregates formed in the presence of Fab against discoidin or gp80 were in slug form by 5 hr. By 10 hr, 30–40% of the aggregates formed in the presence of Fab against gp150 were still unable to form slugs (Table 1). Eventually, 70–80% of these aggregates recovered, forming mature fruiting bodies by 24 hr. Aggregates formed in the presence of anti-gp150 Fab were delayed in their development by ≈5 hr.

DISCUSSION

The use of univalent antibodies in blocking cell reassociation has led to the discovery of a number of cell-surface components that may play a significant role in cell cohesion in *D. discoideum* (5, 6, 14, 21, 22) and in other systems (23–25). In this report, we have made use of this approach and demonstrated that Fab directed against the cell-surface glycoprotein gp150 blocks the cell-sorting process in reconstituted cell aggregates of *D. discoideum*. Antisera raised against purified gp150 or their Fab fragments are effective in blocking cell reassociation mediated by the EDTA-resistant binding sites (14, 26). A more detailed analysis has shown that the Fab fragments affect cell–cell binding at the postaggregation stages but they inhibit poorly at the aggregation stage (15). Using immunohemocyanin labeling of intact aggregates at the slug stage of development, Geltosky *et al.* (27) have clearly demonstrated a nonrandom distribution of gp150 on the cell surface. Molecules of gp150 are clustered at or near sites of cell contact and thus may play a specific role in cell–cell interaction at this stage.

The role of gp150 in cell–cell interactions is clearly shown by the specific inhibitory effect of anti-gp150 Fab on cell sorting. Although the contact site A glycoprotein (gp80) and the lectin discoidin I have been implicated in cell–cell binding, our results showed that Fab directed against either of these surface molecules failed to affect the cell-sorting process or morphogenesis of the reconstituted aggregates. Recently, we have found that anti-gp80 Fab is about 2-fold more effective in inhibiting the EDTA-resistant binding sites of cells at the aggregation stage than at the culmination stage (unpublished data). A surface glycoprotein of M_r 95,000 has also been shown to be responsible for cell–cell binding immediately after the aggregation stage (21, 28). Therefore, it appears that several different and possibly unrelated cell–cell adhesion systems are present on the surface of differentiating amoebae, and the modulation of these systems may enable the cells to undergo different types of cell–cell interaction related to a particular stage.

One possible mechanism of cell sorting is differential cell adhesiveness (29). A recent study by Tasaka and Takeuchi (30) supports the notion that cell sorting among prestalk and prespore cells is mediated by differential cell cohesiveness. We have provided evidence in support of this hypothesis (15). Prespore cells isolated from preculmination- and culmination-stage slugs are significantly more cohesive than prestalk cells and aggregation-stage cells. The kinetics of reassociation among prespore cells are much faster than prestalk cells. Prespore cells are also more resistant to EDTA dissociation and form larger clumps than prestalk cells (15). These observations are consistent with the sorting pattern of prespore and prestalk cells presented in Fig. 1. Prestalk cells move to the outer periphery of the aggregate to envelop the prespore cells, suggesting that the cohesive force among prestalk cells is weaker than that of prespore cells. Subsequently, prestalk cells move to one end of the aggregate, establishing a clear polar organization similar to that in normal pattern formation. However, when cells dissociated from migrating slugs are allowed to reaggregate and sort out, a reverse sorting pattern with prestalk cells enveloped by prespore cells has been reported (12, 30). It is possible that the cohesive forces on prestalk cells and prespore cells are modulated differently during slug migration.

Differential cohesiveness may be regulated by the differential expression of cell-cohesion molecules on the surface. Using FITC-conjugated antibody binding to intact cells and quantitation of the fluorescence in a cell sorter, Geltosky *et al.* (14) detected two cell populations bearing different amounts of gp150 on the surface. Their results suggest that prespore cells may accumulate a higher concentration of gp150 on the surface than prestalk cells. We have found that anti-gp150 Fab is much more effective in blocking the EDTA-resistant binding sites on prespore cells than those on prestalk cells (15). The role of gp150 in prespore cells and prestalk cells may also be modulated by its surface distribution and its microenvironment in the plasma membrane of these two cell types.

We thank Dr. J. Geltosky for providing us with antiserum directed against gp150. This work was supported by a grant from the Medical Research Council of Canada. C.-H.S. is the recipient of a Medical Research Council Scholarship.

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