

# The Contact Site A Glycoprotein Mediates Cell-Cell Adhesion by Homophilic Binding in *Dictyostelium discoideum*

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**Abstract.** *Dictyostelium discoideum* expresses a developmentally regulated cell surface glycoprotein of  $M_r$  80,000 (gp80), which has been implicated in the formation of the EDTA-resistant contact sites A at the cell aggregation stage. To determine whether gp80 participates directly in cell binding and, if so, its mode of action, we conjugated purified gp80 to Covaspheres (Covalent Technology Corp., Ann Arbor, MI) and investigated their ability to bind to cells. The binding of gp80-Covaspheres was dependent on the developmental stage of the cells, with maximal interaction at the late aggregation stage. Scanning electron microscopic studies revealed the clustering of gp80-Covaspheres at the polar ends of these cells, similar to the pattern of gp80 distribution on the cell surface as reported earlier (Choi, A. H. C., and Siu, C.-H., 1987, *J. Cell Biol.*, 104:1375-1387). Precoating cells with an adhesion-blocking anti-gp80 monoclonal antibody

inhibited the binding of gp80-Covaspheres, suggesting that Covasphere-associated gp80 might undergo homophilic interaction with gp80 on the cell surface. Quantitative binding of  $^{125}\text{I}$ -labeled gp80 to intact cells gave an estimate of  $1.5 \times 10^5$  binding sites per cell at the aggregation stage. Binding of soluble gp80 to cells was blocked by precoating cells with the anti-gp80 monoclonal antibody. The ability of gp80 to undergo homophilic interaction was further tested in a filter-binding assay, which showed that  $^{125}\text{I}$ -labeled gp80 was able to interact with gp80 bound on nitrocellulose in a dosage-dependent manner. In addition, reassociation of cells was significantly inhibited in the presence of soluble gp80, suggesting that gp80 has a single cell-binding site. These results are consistent with the notion that gp80 mediates cell-cell binding at the aggregation stage of development via homophilic interaction.

**I**NTERCELLULAR cohesion plays a vital role in the morphogenesis and development of the cellular slime mold *Dictyostelium discoideum*. At the onset of development, EDTA-sensitive-binding sites are expressed rapidly on the cell surface. At 5-6 h of development, cells begin to acquire a sensitivity to extracellular cAMP and undergo chemotactic migration towards areas of higher cAMP concentration (20). At this stage, cells express a number of new antigenic determinants on the cell surface (32-34) and an increase in cell cohesiveness (27). New cell-cell-binding sites that are resistant to EDTA dissociation begin to appear on the cell surface. These binding sites were first defined serologically by Gerisch and co-workers (1, 2) and were named contact sites A. Beug et al. (2) prepared polyclonal antibodies against aggregation-stage cells. After absorption against vegetative cells, Fab fragments derived from these antibodies are capable of inhibiting contact sites A. Subsequently, they found that the inhibitory effect of the Fab fragments can be neutralized by a highly enriched membrane glycoprotein of  $M_r$  80,000 (gp80)<sup>1</sup> (24). Since gp80 contains some highly im-

munogenic carbohydrate moieties, several polyclonal antisera developed against gp80 that can inhibit cell cohesion, show cross-reactivity with other glycoproteins (17, 25, 31). Therefore, it has been enigmatic whether gp80 is actually involved in cell cohesion. To resolve this issue, we have raised a large number of monoclonal antibodies directed against gp80. One of them, 80L5C4, was monospecific for gp80 and it is also capable of blocking the EDTA-resistant contact sites A (31). It is, therefore, very likely that gp80 is directly involved in cell cohesion.

The role of gp80 in cell cohesion is apparently stage specific, since the monoclonal antibody 80L5C4 inhibits the EDTA-resistant binding sites only at the aggregation stage and its effect diminishes rapidly afterwards (31). Synthesis of gp80 occurs only for a discrete period corresponding closely to the expression of contact sites A (16, 25). The amount of gp80 begins to decrease after completion of cell mound formation (25, 26, 31). These observations indicate that gp80 is playing a transient, though crucial, role in cell cohesion during development.

It has been suggested that contact sites A might be involved in the formation of end-to-end contacts among cells (13). Cells at the aggregation stage are more elongated and the cell surface expresses a large number of filopodia, which are characteristic of early migrating cells (8). Surface localiza-

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1. *Abbreviations used in this paper:* gp, glycoprotein; N-CAM, neuronal cell adhesion molecule; SEM, scanning electron microscopy.

tion by immunofluorescence labeling or immunoelectron microscopy reveals a biased distribution of gp80 on these cells (4). gp80 molecules are more abundantly localized at the two polar ends of cells and on the filopodia, suggesting that they are important in mediating the formation of end-to-end contacts between cells.

Although it is evident that gp80 plays an important role in cell cohesion in *D. discoideum*, several vital questions remain to be answered. Does gp80 function in a complex comprised of the bona fide binding molecule or does it mediate cell-cell binding directly? If the latter is true, how does it mediate cell-cell binding? In the present report, we have developed several in vitro assays to address these questions. Purified gp80 is capable of binding to cells in a stage-specific manner. gp80 binding can be inhibited by precoating cells with the monoclonal antibody 80L5C4. Soluble gp80 binds quantitatively to gp80 immobilized on nitrocellulose membrane. In addition, cell reassociation is inhibited significantly in the presence of soluble gp80. Results of these studies support the notion that the EDTA-resistant type of cell-cell binding at the aggregation stage is mediated by homophilic interaction between gp80 molecules on adjacent cells.

## Materials and Methods

### Cell Strain and Culture Conditions

The wild type strain NC4 of *Dictyostelium discoideum* was used in all experiments and the axenic strain AX2 was used for the purification of gp80. NC4 cells were cultured in association with *Klebsiella aerogenes* and were developed as described (39). Growth phase cells were collected from the partially cleared bacterial lawn, washed free of bacteria, and plated at  $2 \times 10^8$  cells/ml on filter paper (No. 50; Whatman, Inc., Clifton, NJ) for development. Under these conditions, cells began to aggregate between 6 and 8 h and aggregation was completed by 12 h with the formation of round mounds. Culmination began at  $\sim 18$  h. Alternatively, cells were resuspended at  $10^7$  cells/ml in 17 mM phosphate buffer, pH 6.4, and shaken at 180 rpm for development. The axenic strain AX2 was cultured in HL-5 medium as described by Cocucci and Sussman (5). Cells were collected in their late exponential growth phase and developed in 17 mM phosphate buffer, pH 6.4, for 12 h, with cAMP pulsing ( $2 \times 10^{-8}$  M final concentration) at 7-min intervals.

### Purification of gp80

gp80 was purified from AX2 cells after 12 h of development in liquid culture. Cells were collected and homogenized. The particulate fraction was solubilized with 1% sodium cholate and 0.2% NP-40 in PBS. The sample was centrifuged at 100,000 g for 60 min and the soluble fraction was decanted and diluted with PBS at 1:1 ratio. The sample was loaded on to a concanavalin A (Con A) column and bound glycoproteins were eluted with 0.1 M  $\alpha$ -methyl-mannoside in 0.1% NP-40 or 0.1% Triton X-100. Fractions were pooled and dialyzed before being chromatographed on a DEAE-cellulose column, equilibrated in 10 mM sodium acetate, pH 5.5, 0.5 mM EDTA, and 0.1% Triton X-100. The column was eluted with a linear gradient of 0 to 150 mM NaCl. Fractions enriched in gp80 were pooled, and dialyzed, and then adsorbed onto an affinity column prepared by coupling 80L5C4 IgG to CNBr-activated Sepharose 4B (Pharmacia, Inc., Piscataway, NJ). The bound protein was eluted with 50 mM diethylamine, pH 11.3. Protein fractions were neutralized immediately and dialyzed against PBS or 0.1% octyl glucoside in PBS. Purity of the gp80 sample was evaluated by silver staining (23) after electrophoretic separation in polyacrylamide gel (15). Homogeneity was achieved usually by one passage through the immunoaffinity column. Purified gp80 was stored either in lyophilized form or in solution at  $-70^\circ\text{C}$ .

### Coupling of gp80 to Covaspheres

Green or red MX Covaspheres (approximately 0.5  $\mu\text{m}$  in diameter) were obtained from Covalent Technology Corp. (Ann Arbor, MI). Covaspheres

were first sonicated for 1 min to disperse the aggregates and then 10  $\mu\text{g}$  of purified gp80 was added to 0.1 ml of Covaspheres. The final volume was adjusted to 0.2 or 0.3 ml with PBS. The mixture was incubated on a rotator for 75 min at room temperature and the Covaspheres were pelleted by centrifugation. The supernate was discarded and the pellet was resuspended in 1 ml of 1% BSA in PBS by brief sonication. This was repeated once and the gp80-conjugated Covaspheres were stored in suspension in 0.2 ml of 1% BSA at  $4^\circ\text{C}$ . Coupling of Con A, BSA, or protein A to Covaspheres was carried out using the same procedure.

### Binding of gp80-Covaspheres to Cells

NC4 cells were collected from filter pads at different stages of development. Cells were dissociated mechanically and washed in 17 mM phosphate buffer, pH 6.4. Cells were resuspended at  $5 \times 10^6$  cells/ml in phosphate buffer containing 5 mM EDTA to block the EDTA-sensitive-binding sites. gp80-Covaspheres were sonicated and washed once in 17 mM phosphate buffer before addition to the cell suspension at 1:20 dilution. Samples were vortexed and rotated on a platform shaker at 180 rpm at  $4^\circ\text{C}$ . Since small aggregates of Covaspheres began to appear after 60 min under these conditions, binding studies were routinely carried out for 20–30 min before microscopic observation. To cut down the background of unbound Covaspheres, cells were pelleted by brief centrifugation and the unbound Covaspheres in the supernate were removed. The loose pellet was resuspended gently for microscopic observation or further diluted 10-fold for quantitative analysis in a cell sorter (model V; Epic Inc., NY). For each sample, 10,000 cell particles were counted and subdivided into single cell and aggregate channels for further analysis.

### Scanning Electron Microscopy (SEM)

Cells were deposited on cover glass with 0.1% poly-L-lysine (Sigma Chemical Co., St. Louis, MO) (22). After 8–10 h of development, cells were incubated with gp80-Covaspheres for 10 min on a platform shaker rotated at 60 rpm. Unbound Covaspheres were quickly rinsed off and cells were fixed with 2% glutaraldehyde for 30 min at room temperature. Subsequent steps of dehydration and critical point drying were carried out as previously described (4). Samples were observed with a Jeol JSM 840 scanning electron microscope.

### Binding of gp80 to Intact Cells

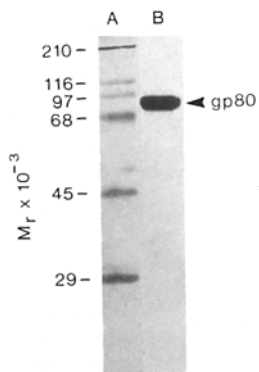
Immunoaffinity-purified gp80 was dialyzed against 0.1% octyl glucoside in PBS. gp80 was radioiodinated using the chloramine T method as previously described (32). Different concentrations of  $^{125}\text{I}$ -labeled gp80 were prepared in phosphate buffer immediately before addition to cell suspension, with a final detergent concentration not exceeding 0.005%. Cells were resuspended in 10 mM EDTA and 17 mM phosphate buffer, pH 6.4, at  $2-5 \times 10^6$  cells/ml. Incubation was carried out at  $4^\circ\text{C}$  for 45 min on a platform shaker. Cell samples were layered on top of 0.3 ml of an oil mixture containing bis-(2-ethylhexyl)-phthalate and *n*-butyl phthalate (1:1.2, vol/vol), and then centrifuged to separate the unbound gp80 from the cells. The cell pellet was counted in a gamma counter and the amount of gp80 bound was estimated. The amount of nonspecifically bound gp80 was estimated by including a 40-fold higher concentration of unlabeled gp80 in the incubation mixture. This value was subtracted from the total amount of radioactivity bound to cells.

### Competition of gp80 Binding to Cells by Monoclonal Antibody

Cells were resuspended at  $2 \times 10^7$  cells/ml in 17 mM phosphate buffer, pH 6.4, containing 5 mM EDTA and then precoated with 80L5C4 IgG (31) at 10  $\mu\text{g}/\text{ml}$  for 15 min at  $4^\circ\text{C}$ . Cells were washed once and resuspended at  $5 \times 10^6$  cells/ml in EDTA/phosphate buffer containing 0.25 mg/ml goat anti-mouse IgG Fab (Cappel Laboratories, Inc., Cochranville, PA) for 15 min at  $4^\circ\text{C}$ . The sample was briefly vortexed before the addition of gp80-Covaspheres or labeled gp80. Subsequent steps were carried out in accordance with the corresponding assay protocol.

### Filter-binding Assay

gp80 samples were spotted on to nitrocellulose disks, which were blocked with 5% skim milk in PBS and 0.05% Tween-20 and then incubated with different amounts of  $^{125}\text{I}$ -labeled gp80 for 30 min at room temperature. A blank disk was included in each sample for background estimation. At the end of the incubation period, disks were washed with at least three changes of



**Figure 1.** PAGE of gp80. gp80 was purified on an immunoaffinity column as described in Materials and Methods. Purified gp80 was subjected to gel electrophoresis according to Laemmli (15) and the protein was detected by silver staining (23). (A) Molecular mass markers; (B) 5  $\mu$ g gp80.

5% skim milk followed by two changes of 0.05% Tween-20 in PBS. Disks were air dried and counted in a gamma counter. In competition experiments, nitrocellulose disks were preincubated with different amounts of unlabeled gp80 for 5 min before the addition of a constant amount of labeled gp80. Binding was carried out for another 30 min. Disks were washed and processed for counting.

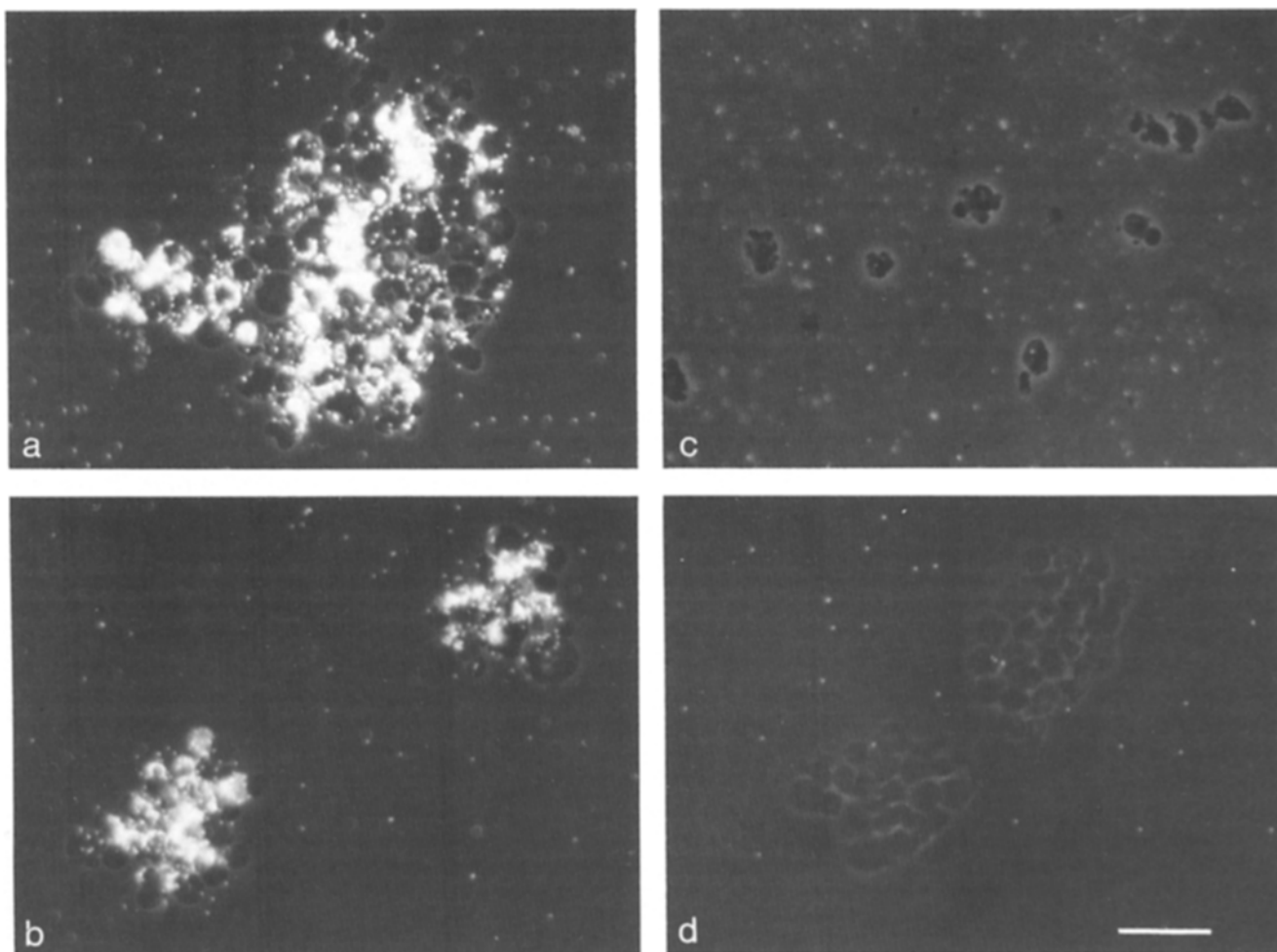
### Cell Cohesion Assay

Cell-cell adhesion was assayed as described previously (31). Cells were dissociated and suspended at  $2-3 \times 10^6$  cells/ml in 17 mM phosphate buffer, pH 6.4, with 5 mM EDTA. Samples were rotated vertically on a platform shaker at 180 rpm. Cells were counted at different time intervals using a hemacytometer. Both singlets and doublets were scored as unaggregated cells.

### Results

#### Binding of gp80-conjugated Covaspheres to Cells

Immunoaffinity-purified gp80 was analyzed by gel electrophoresis followed by silver staining. Fig. 1 shows that no detectable contaminating components were present in the sample. The gp80 preparation was considered pure and was used for conjugation to Covaspheres. gp80-Covaspheres were sonicated to disperse aggregates before use. NC4 cells at 12 h of development were dissociated into single cells and then resuspended in phosphate buffer containing 5 mM EDTA. The appropriate amount of gp80-Covaspheres was



**Figure 2.** Binding of gp80-Covaspheres to aggregation-stage cells. NC4 cells were developed in liquid medium for 12 h and then dissociated into single cells in 20 mM EDTA. Cells were resuspended at  $5 \times 10^6$  cells/ml and incubated with Covaspheres at 4°C in 17 mM phosphate buffer, pH 6.5, containing 5 mM EDTA. Samples were observed under a fluorescence microscope after 20 min. (a and b) Cells were incubated with gp80-Covaspheres. (c) Cells were precoated with 80L5C4 IgG and goat anti-mouse IgG Fab before the addition of gp80-Covaspheres. (d) Cells were incubated with BSA-Covaspheres. Bar, 20  $\mu$ m.

**Table I. Binding of GP80-conjugated Covaspheres to NC4 Cells**

Cell stage	Type of Covaspheres	Cell particles with Covaspheres*	
		Single cells	Aggregates
<i>h</i>		%	%
0	gp80-Covaspheres	6.7 ± 0.9	ND†
0	Con A-Covaspheres	62.4 ± 2.8	ND
12	BSA-Covaspheres	10.7 ± 2.5	24.0 ± 1.7
12	Protein A-Covaspheres	8.0 ± 2.0	21.7 ± 4.0
12	gp80-Covaspheres	49.3 ± 6.5	71.0 ± 7.5
12	80L5C4 IgG + gp80-Covaspheres <sup>  </sup>	13.0 ± 2.0	ND
12	Trypsin-treated gp80-Covaspheres <sup>†</sup>	16.6 ± 2.3	29.1 ± 3.0
12	80L5C4-coated gp80-Covaspheres**	13.4 ± 2.7	20.9 ± 4.6
12	Con A-Covaspheres	68.7 ± 4.5	78.7 ± 7.8
12	80L5C4 IgG + Con A-Covaspheres	59.8 ± 2.9	ND

The binding of Covaspheres to 0 and 12 h NC4 cells was carried out in 5 mM EDTA and 17 mM phosphate buffer, pH 6.4, as described in Materials and Methods. Samples were analyzed in an EPICS V cell sorter. 10,000 particles were counted for each sample and the data were subjected to computer analysis.

\* Particles were separated into two categories, single cells and aggregates. Values represent the percentage of fluorescent particles to the total number of particles under each category. Results are expressed as mean ± SD of three determinations.

† ND, not determined. Greater than 95% of cells in these samples remained as single cells.

|| Cells were precoated with 80L5C4 IgG at 10 µg/ml for 15 min and then resuspended in goat anti-mouse IgG Fab (0.2 mg/ml) and 5 mM EDTA. The cells were briefly vortexed and then allowed to bind with either gp80-Covaspheres or Con A-Covaspheres at 4°C.

† gp80-Covaspheres were digested with trypsin at 0.1 mg/ml for 15 min at 37°C and the reaction was terminated by the addition of an equivalent amount of trypsin inhibitor. The spheres were washed twice with phosphate buffer before being used in the binding assay.

\*\* gp80-Covaspheres were precoated with 80L5C4 Fab at 0.5 mg/ml for 30 min at room temperature and the unbound Fab was removed before being used in the binding assay.

added and the sample was rotated on a platform shaker at 4°C. About 40% of the cells reformed small aggregates after 20 min of incubation. By that time, a large number of gp80-Covaspheres had bound to the cell aggregates (Fig. 2, *a* and *b*). In the control experiment, cells were mixed with BSA-conjugated Covaspheres and a maximum of two to three Covaspheres per aggregate was observed (Fig. 2 *d*). Therefore, nonspecific trapping of Covaspheres in cell aggregates appeared to be minimal.

To quantitate the binding of Covaspheres to cells, samples were subjected to analysis in a cell sorter. Cell particles were divided into two categories, single cells and aggregates, and the percentage of cell particles that carried fluorescein-labeled spheres was estimated. The proportion of cell particles with bound gp80-Covaspheres was almost five times higher than the control using BSA-Covaspheres in the single cell category and more than three times higher in the aggregate category (Table I). As an additional negative control, protein A was conjugated to Covaspheres and allowed to bind to 12-h cells. The percentage of cells with bound Covaspheres was similar to the BSA control (Table I). In both controls, cell aggregates appeared to trap Covaspheres more easily. As a positive control, the binding of Con A-conju-

gated Covaspheres to 0 and 12 h cells was performed. Con A-Covaspheres bound equally well to cells of both stages. For the 12-h sample, the percentages of cell particles with bound Con A-Covaspheres was comparable to the case of gp80-Covaspheres. gp80-Covaspheres did not bind significantly to 0-h cells (Table I).

### Homophilic Binding of gp80

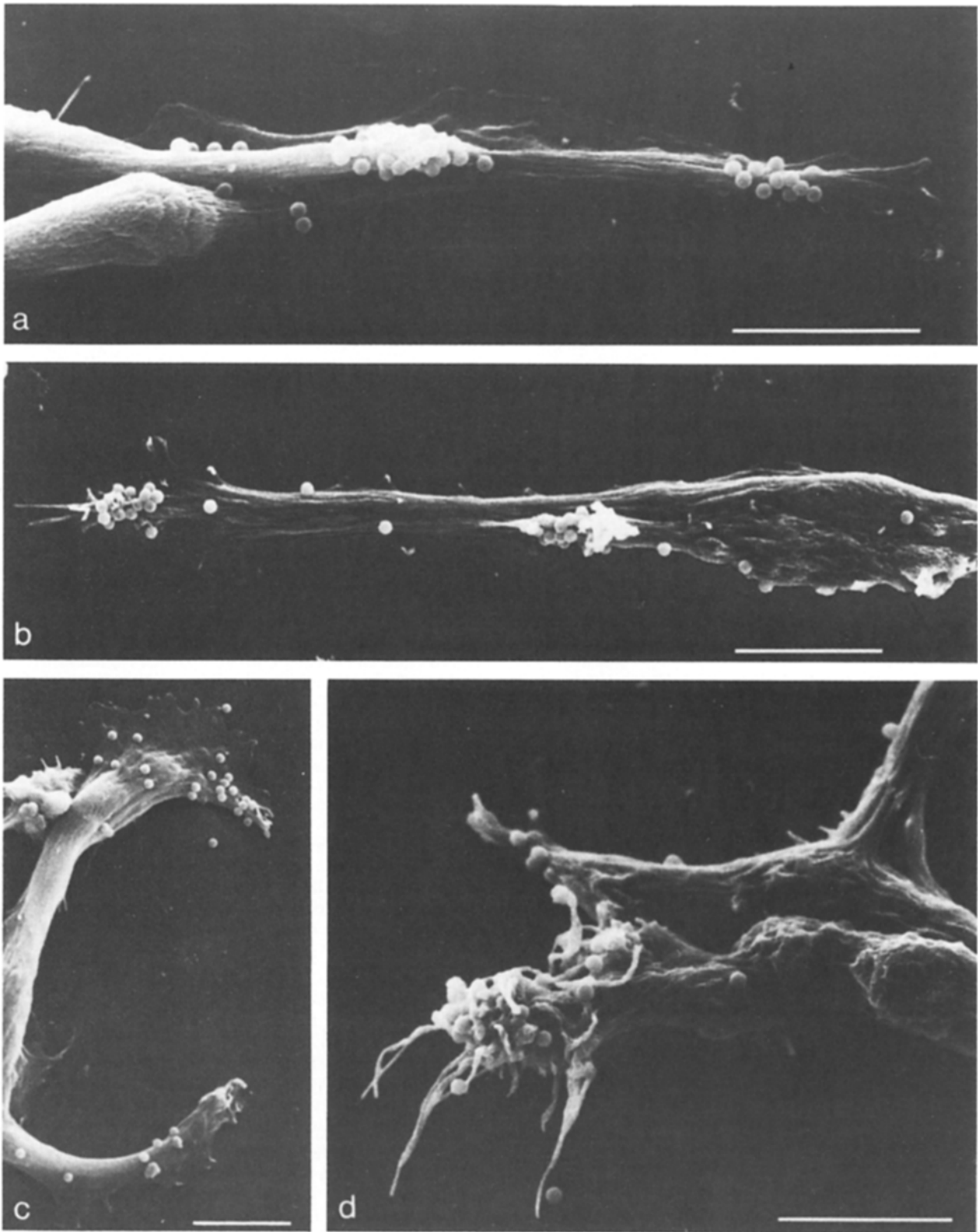
To determine whether gp80 binds homophilically to gp80 molecules on the cell or heterophilically to other surface receptor(s), 12-h cells were precoated with the monoclonal antibody 80L5C4, which blocked the cell-binding site of gp80 (32). The cells were further coated with goat anti-mouse IgG Fab and then incubated with gp80-Covaspheres. Fig. 2 *c* shows that the cells were effectively dissociated by the monoclonal antibody and most of the cells did not have a single sphere bound on the surface. Quantitative analysis showed that the percentage of cells with bound spheres dropped to background level (Table I). As a control, cells with or without precoating with 80L5C4 IgG and the second univalent antibody were allowed to bind Con A-Covaspheres. About 60% of cell particles contained Con A-Covaspheres in both cases (Table I). To demonstrate that the binding of Covaspheres to cells was dependent on gp80, gp80-Covaspheres were treated with trypsin before the binding assay. Results in Table I show that the binding of trypsinized gp80-Covaspheres to cells dropped to background level. When gp80-Covaspheres were pretreated with 80L5C4 Fab, binding of Covaspheres to cells was also blocked (Table I). These results suggest that the gp80 conjugated on Covaspheres binds homophilically to the cell surface gp80 and that the binding is dependent on the availability of the cell-binding site on gp80.

### Localization of gp80-Covaspheres by SEM

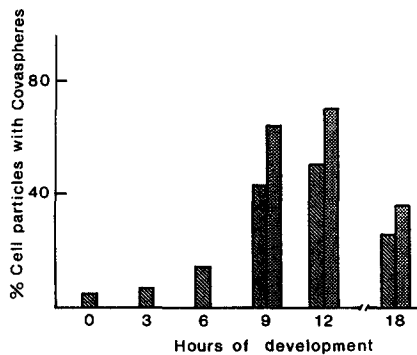
We have previously shown that gp80 is preferentially localized at the two polar ends of the elongated migration-stage cell (4). It is, therefore, expected that the binding of gp80-Covaspheres to these cells should reflect the physiological distribution of gp80 on the cell surface. To test this, cells were developed on polylysine-coated coverslips and gp80-Covaspheres were added to the medium at the midaggregation stage for 10 min to allow interaction with the migrating cells. Samples were immediately fixed and processed for SEM. gp80-Covaspheres showed a clearly biased distribution on the cell surface. Covaspheres appeared to cluster mainly at the polar ends of these elongated cells (Fig. 3, *a* and *b*). They were also observed on lamellipodia as well as filopodia (Fig. 3, *c* and *d*). The control using BSA-Covaspheres showed only sparse binding while the binding of Con A-Covaspheres to the cell surface had a random distribution (data not shown).

### Stage-specific Binding of gp80-Covaspheres to Cells

Since the expression of gp80 is developmentally regulated (25, 31), the binding of gp80-Covaspheres to cells in the developmental cycle should reflect stage specificity corresponding to the time of gp80 appearance on the cell surface. To investigate this, cells were developed for different time periods and collected for binding studies. Fig. 4 shows that gp80-Covaspheres did not bind significantly to vegetative



**Figure 3.** Scanning electron micrographs showing the distribution of gp80-Covaspheres of the cell surface. Cells were developed on coverglass coated with 0.1% poly-L-lysine for 12 h. 20  $\mu$ l of gp80-Covaspheres was added to 1 ml of development medium in the petri dish that contained the coverglass. The sample was rotated on a platform shaker at 60 rpm for 10 min. The unbound Covaspheres were washed off and the sample was fixed with glutaraldehyde and processed for SEM. (a and b) Elongated cells in a migrating stream, showing the clustering of gp80-Covaspheres with filopodia. (c) Association of gp80-Covaspheres with lamellipodia. (d) Association of gp80-Covaspheres with filopodia. Bars, 5  $\mu$ m.



**Figure 4.** Binding of gp80–Covaspheres to cells at different developmental stages. NC4 cells were developed on filter pad and collected for the Covasphere-binding assay at different stages. Binding of gp80–Covaspheres to cells was carried out as described under Table I. As a positive control for vegetative-stage cells, a sample was incubated with Con A–Covaspheres. (Hatched bar) Single cell particles; (stippled bar) particle of aggregates.

cells or preaggregation-stage cells, although two-thirds of the cell population at 0 h bound Con A–Covaspheres (Table I). The number of cells that bound gp80–Covaspheres increased rapidly between 6 and 9 h of development, corresponding to the time when gp80 is known to be actively synthesized in the cell (17, 25). At 18 h, the number of cells with Covaspheres dropped by ~50%.

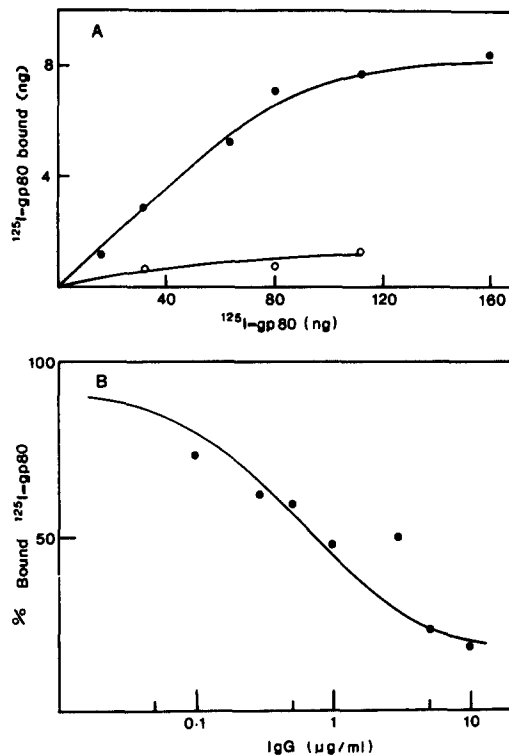
#### Binding of $^{125}\text{I}$ -Labeled gp80 to Intact Cells

The binding of gp80 to cells was also carried out using  $^{125}\text{I}$ -labeled gp80. Since gp80 formed aggregates in solution, gp80 prepared in detergent-free buffer bound to cells poorly and results were often inconsistent. When samples were dialyzed against 0.1% octyl glucoside before use, gp80 molecules were capable of binding to cells. Cells were incubated with the  $^{125}\text{I}$ -labeled gp80 for 45 min on a platform shaker. The assay was carried out at 4°C in the presence of 10 mM EDTA to slow down the kinetics of cell reassociation. Under these conditions, usually <40% of the cells formed small aggregates at the end of the incubation period, thus minimizing nonspecific trapping of gp80. Usually between 80 and 90% of the total bound  $^{125}\text{I}$ -gp80 could be competed off by preincubation with a 40-fold concentration of unlabeled gp80. Fig. 5 shows that the binding of  $^{125}\text{I}$ -labeled gp80 to cells was both dosage dependent and saturable. The number of gp80-binding sites on the cell surface was estimated to be  $\sim 1.5 \times 10^5$  sites/cell. In addition, the binding of  $^{125}\text{I}$ -labeled gp80 to 3-h cells was only at background level, indicating stage specificity of the binding reaction (Fig. 5 a).

The effect of precoating cells with the anti-gp80 monoclonal antibody 80L5C4 was also examined. The binding of  $^{125}\text{I}$ -labeled gp80 to cells was inhibited in a dosage-dependent manner (Fig. 5 b). These results are consistent with the notion that the soluble gp80 binds homophilically with the membrane-bound gp80 of aggregation-stage cells.

#### gp80-mediated Aggregation of Covaspheres

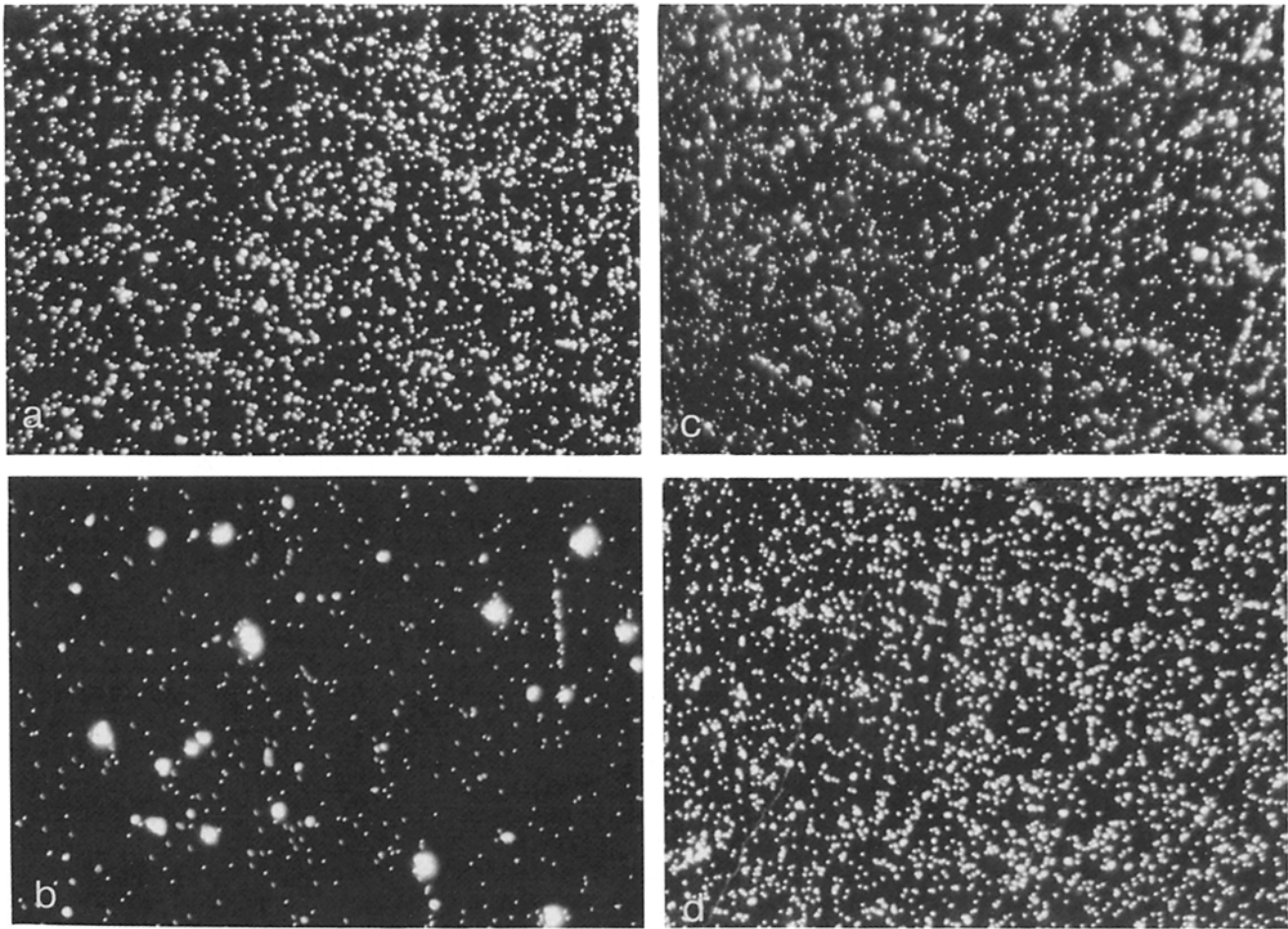
In the cell-binding experiment using gp80–Covaspheres, we noted that small aggregates of gp80–Covaspheres began to appear after 60 min of incubation on a platform shaker. The



**Figure 5.** Binding of  $^{125}\text{I}$ -labeled gp80 to cells. Purified gp80 was dialyzed against 0.1% octyl glucoside and labeled with  $^{125}\text{I}$  using chloramine T. Cells were developed in liquid medium for 12 h and then collected for the assay. Cells were washed and resuspended in 5 mM EDTA and phosphate buffer at  $4 \times 10^6$  cells/ml. (A) Samples of 0.1 ml of cell suspension were incubated with different amounts of  $^{125}\text{I}$ -labeled gp80 (sp act = 1,560 cpm/ng) for 45 min at 4°C. For background estimation, a 40-fold higher concentration of unlabeled gp80 was included in each corresponding sample. Background radioactivity was subtracted from the total bound radioactivity for each sample. (●) 12-h cells, (○) control using 3-h cells without background subtraction. (B) Inhibition of  $^{125}\text{I}$ -labeled gp80 binding was carried out by precoating the cell surface gp80 with different amounts of 80L5C4 IgG and 0.2 mg/ml goat anti-mouse IgG Fab before the addition of 64 ng of  $^{125}\text{I}$ -labeled gp80 to each sample. Results are presented as the percentage of the amount of labeled gp80 bound in the control sample.

aggregation of gp80–Covaspheres was further investigated. Covaspheres conjugated with gp80 or protein A were first sonicated to disperse all aggregates (Fig. 6 a) and then gyrated on a platform shaker at 200 rpm and samples were observed under the fluorescence microscope at regular intervals. Large aggregates of gp80–Covaspheres were observed at 90 min (Fig. 6 b), while protein A–Covaspheres and BSA–Covaspheres did not show any significant degree of self-aggregation under similar conditions (data not shown). When gp80–Covaspheres were rotated in the presence of soluble gp80, self-association was inhibited (Fig. 6 c). The data clearly indicate that the reassociation of gp80–Covaspheres was mediated by homophilic interaction of the gp80 molecules conjugated on these spheres. To further demonstrate that the reassociation of gp80–Covaspheres was dependent on the cell-binding site of gp80 molecules, gp80–Covaspheres were rotated in the presence of Fab fragments





**Figure 6.** Epifluorescence micrographs showing reaggregation of gp80-Covaspheres. gp80-Covaspheres were sonicated briefly to disperse all aggregates and then allowed to reaggregate on a platform shaker (200 rpm) at room temperature. One sample was incubated in the presence of 80L5C4 Fab at 1 mg/ml. Samples were examined at different intervals under the microscope. (a) gp80-Covaspheres right after sonication. (b) Formation of aggregates of gp80-Covaspheres at 90 min. (c) Inhibition of self-aggregation in the presence of soluble gp80 (25 µg/ml in 0.05% octyl glucoside) at 90 min. (d) Inhibition of Covasphere reaggregation in the presence of 80L5C4 Fab (0.5 mg/ml) at 90 min.

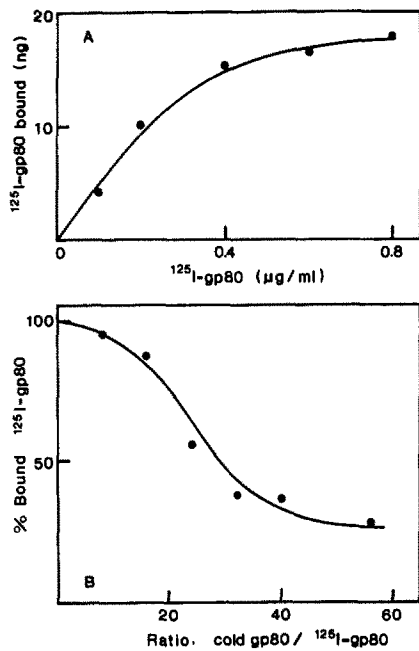
derived from 80L5C4 IgG. Reassociation of gp80-Covaspheres was completely abolished (Fig. 6 d). When the sample was examined after 90 min, 3 and 15 h of rotation, the gp80-Covaspheres remained as single particles.

#### **Binding of <sup>125</sup>I-Labeled gp80 to Filter-bound gp80**

The interaction of gp80 with gp80 was also studied using an *in vitro* filter-binding assay. Unlabeled gp80 was immobilized on nitrocellulose membrane and incubated with <sup>125</sup>I-labeled gp80. Quantitative binding between the soluble gp80 and the immobilized gp80 was analyzed. Fig. 7 shows the dosage-dependent-binding curve of gp80-gp80 interaction. At the saturation level, ~1 mol of immobilized gp80 bound 0.2 mol of soluble gp80, suggesting that the binding of gp80 to nitrocellulose probably rendered 80% of the gp80-binding sites unavailable for interaction. To demonstrate the binding specificity, competition experiments were carried out. Inclusion of a 50-fold higher concentration of unlabeled gp80 reduced the binding of <sup>125</sup>I-labeled gp80 to nitrocellulose-bound gp80 by ~80% (Fig. 7 B).

#### **Effect of Soluble gp80 on Cell-Cell Binding**

If solubilized gp80 exists in monomeric form and each monomer contains only one cell-binding site, binding of soluble gp80 to cells should block cell-cell adhesion mediated by the homophilic interaction of membrane-associated gp80 molecules. Such an experiment was performed with aggregation-stage cells. gp80 was dialyzed in 0.1% octyl glucoside and then added to cells to give a final detergent concentration of 0.005%. The mixture was vortexed for 10 s to dissociate cells and the reassociation of cells was monitored under microscope at regular intervals. Cell reassociation was significantly inhibited in the presence of soluble gp80 during the first 45 min of incubation, while cells incubated in the presence of an equivalent amount of octyl glucoside showed the normal kinetics of reassociation (Fig. 8 A). Cells reassociated in the presence of soluble gp80 formed only small aggregates (Fig. 9). The inhibitory effect of gp80 was dosage dependent (Fig. 8 B). At 10 µg/ml of gp80, ~60% of the cells failed to form aggregates and the effect was halved at 5 µg/ml. The effect of soluble gp80 be-



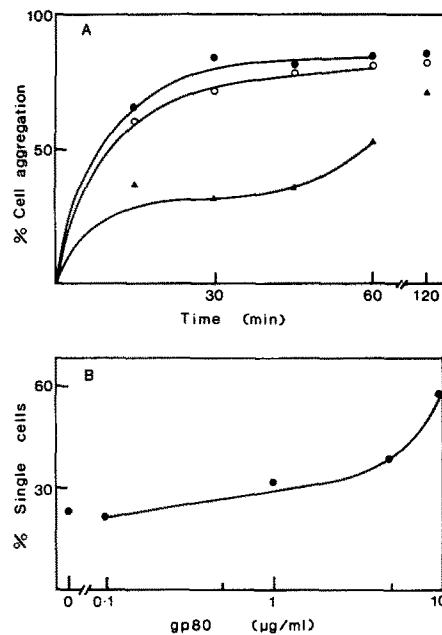
**Figure 7.** Binding of soluble  $^{125}\text{I}$ -labeled gp80 to gp80 immobilized on nitrocellulose membrane. Samples of  $0.1\ \mu\text{g}$  of unlabeled gp80 were immobilized on nitrocellulose discs. After blocking with 5% skim milk, samples were incubated with different concentrations of labeled gp80. Background binding was determined by inclusion of a blank disk in the incubation mixture and was subtracted from the radioactivity bound on the corresponding disk. In competition experiments, filter disks were incubated with a mixture of labeled and unlabeled soluble gp80 at different ratios, with the concentration of the labeled gp80 kept at  $200\ \text{ng/ml}$ . (A) Dosage-dependent binding curve. (B) Competitive inhibition by unlabeled soluble gp80.

came negligible at a concentration of  $0.1\ \mu\text{g/ml}$ . Generally, after 60 min of incubation, cells began to reassociate into larger aggregates even in the presence of  $10\ \mu\text{g/ml}$  of soluble gp80. By 120 min, the degree of reassociation was indistinguishable from that of the control (Fig. 8 A). It is likely that some of the gp80 molecules in solution began to aggregate on the cell surface as a result of the much reduced detergent concentration and this eventually led to the cross-linking of cells.

### Discussion

In this report, results of several different assays clearly demonstrate that purified gp80 is capable of binding specifically to *D. discoideum* cells at the aggregation stage of development. Purified gp80, when conjugated to Covaspheres, can mediate the binding of these spheres to cells in a stage-specific manner. gp80 has a tendency to aggregate in the absence of detergent and it does not bind to surface receptors in a consistent manner. However, prior treatment with a mild detergent allows gp80 to interact with surface components. Of several detergents we have tried, octyl glucoside has the least deleterious effect on cells.

Cells precoated with the anti-gp80 monoclonal antibody 80L5C fail to bind soluble gp80 or gp80-Covaspheres (Fig. 5 and Table I). If gp80-Covaspheres are treated with trypsin or precoated with 80L5C4 Fab, binding of gp80-Cova-

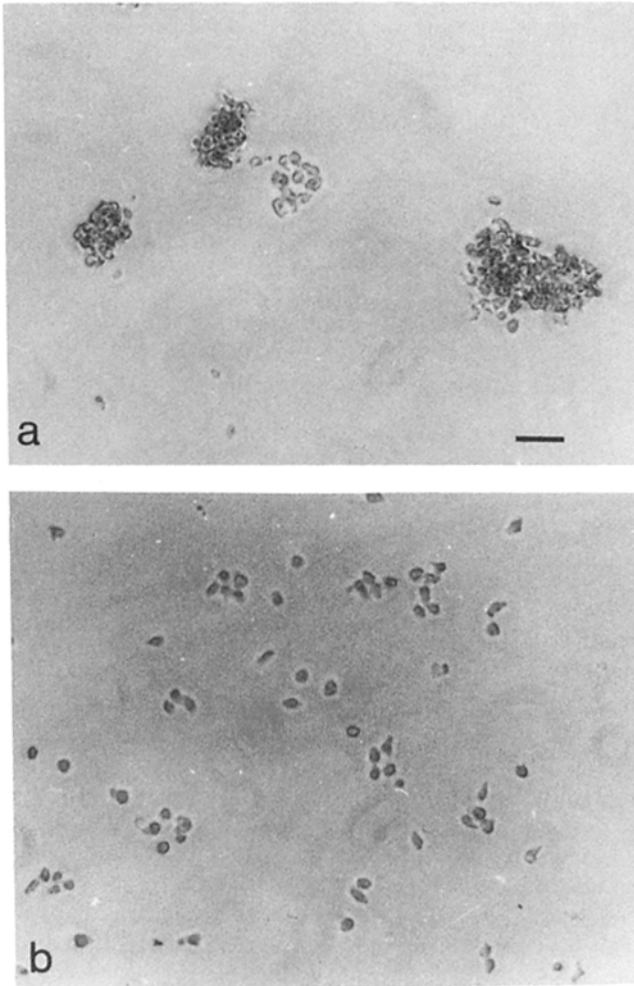


**Figure 8.** Inhibition of cell reassociation by soluble gp80. 10-h NC4 cells were suspended in 5 mM EDTA and 17 mM phosphate buffer, pH 6.4, at  $2.5 \times 10^6$  cells/ml. At 0 min, either detergent or gp80 was added to the sample and cells were vortexed for 10 s to disperse all aggregates. Samples were rotated at 180 rpm on a platform shaker for cells to reassociate. Cell aggregation was monitored at regular intervals using a hemacytometer. (A) Cells were reassociated in the presence of soluble gp80 at  $10\ \mu\text{g/ml}$  ( $\blacktriangle$ ), one control sample was reassociated in the presence of 0.005% octyl glucoside ( $\circ$ ), and the other control was reassociated simply in EDTA-phosphate buffer ( $\bullet$ ). (B) Cell samples were reassociated in the presence of different concentrations of gp80 and the detergent content was kept at 0.005% in all samples. The data were taken from the 30-min point.

spheres to cells is also inhibited (Table I). These results clearly indicate that the interaction of cells with Covaspheres is dependent on the gp80-binding site, and they are consistent with the idea that gp80 molecules undergo homophilic interaction. However, it is inherent in studies using monoclonal antibodies as blocking agents that the results do not usually rule out the possibility of nonspecific steric hindrance. In this case, one can argue that gp80 receptors are closely associated with gp80 molecules on the cell surface and the binding of 80L5C4 IgG to gp80 sterically blocks the accessibility of the receptor's binding site. Two in vitro assays have been developed to resolve this issue. The first assay addresses the question whether Covaspheres conjugated with gp80 can undergo self-association. gp80-Covaspheres are found to reassociate into small aggregates within a short period of time after dispersion. This self-association can be abolished by the inclusion of 80L5C4 Fab in the sample (Fig. 6). In the filter-binding assay, labeled gp80 binds to nitrocellulose-bound gp80 quantitatively and in a saturable manner. The binding can be competed off specifically by unlabeled gp80 (Fig. 7). These results thus indicate that purified gp80 is capable of undergoing homophilic interaction.

It is likely that gp80 behaves in the same manner in vivo. Since the monoclonal antibody 80L5C4 inhibits the binding of soluble gp80 to cells, gp80-Covasphere aggregation, and

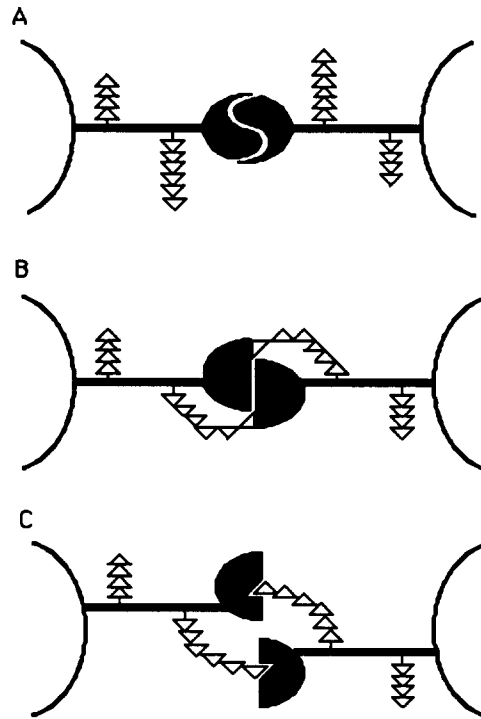




**Figure 9.** Phase micrographs of cells reassociated in the presence or absence of soluble gp80. 10-h cells were reassociated in EDTA-phosphate buffer as described in the legend to Fig. 8 and phase micrographs were taken at 30 min of reassociation. (a) Cells reassociated in 0.005% octyl glucoside, and (b) cells reassociated in soluble gp80 at 10 µg/ml. Bar, 20 µm.

gp80-gp80 interaction at concentrations similar to those required for the disruption of cell-cell contact formation, it suggests that the same gp80-binding site is involved in both *in vivo* and *in vitro* assays. Moreover, when dispersed cells are incubated in the presence of soluble gp80, cell reaggregation is drastically reduced. This result clearly supports the mechanism of homophilic interaction and it also indicates that each gp80 molecule has a single cell-binding site.

It is of interest to note that gp80 and the neuronal cell adhesion molecule (N-CAM) share a number of similarities. They are both involved in the calcium-independent type of cell-cell binding (3). N-CAM also behaves as a ligand in cell-cell adhesion (28). The N-CAM-binding site is located in the amino terminus of the polypeptide chain (6, 7, 11). N-CAM is heavily glycosylated and its embryonic form contains a large amount of sialic acid, resulting in a highly negatively charged molecule (14). The carbohydrate moieties, located primarily in the middle portion of the molecule, are probably not directly involved in cell-cell binding, although



**Figure 10.** Models of gp80-gp80 interaction. (A) gp80-gp80 binding is mediated by homophilic interaction of their protein-binding site, which can be disrupted by 80L5C4 Fab. (B) Each gp80 molecule has a carbohydrate-protein interacting site and a protein-protein interacting site. (C) gp80 acts as a lectin and its own carbohydrate moiety serves as the ligand. (Open triangles) Carbohydrate moieties extending from the polypeptide backbone.

the affinity of N-CAM to N-CAM interaction is apparently modulated by the sialic acid (10). gp80 is also glycosylated, containing at least two types of carbohydrate side-chains, one of which is heavily sulfated (37). Although it has been suggested that the carbohydrate moiety of gp80 may play a role in cell-cell binding (21, 31, 35), it remains to be determined whether it is directly involved in cell binding.

Fig. 10 depicts three possible models for gp80-gp80 interaction. Since the cell-binding activity of gp80 is disrupted by 80L5C4, which recognizes a peptide epitope (31); gp80-gp80 binding can be mediated by homophilic interaction of the protein-binding site (Fig. 10 A). In this case, carbohydrate moieties do not participate in the binding mechanism, but it may modulate the binding affinity as in the case of N-CAM (10). The second model depicts a carbohydrate-protein-interacting site in addition to the protein-protein binding site (Fig. 10 B). This should accommodate the finding that certain antibodies recognizing oligosaccharides also interfere with cell-cell adhesion (31, 35). The third model suggests that gp80 acts as a lectin and its own carbohydrate side-chain serves as the ligand. Since precoating cells or Cova-spheres with 80L5C4 Fab disrupts the adhesive interaction, this model would require that gp80 from both surfaces are allowed to bind with its ligand to achieve stable binding (Fig. 10 C). Future studies on the gp80-binding site should help us determine the precise components involved and the molecular nature of the homophilic interaction.

The role of gp80 in cell cohesion is unique to the aggrega-

tion stage of development. Binding of gp80-Covaspheres corresponds closely to the time when cells express gp80. Maximal expression of gp80 on the cell surface occurs between 10 and 12 h of development. The binding of soluble gp80 to 12 h cells gives an estimate of  $1.5 \times 10^5$  binding sites per cell. This figure agrees closely with the number of gp80 molecules per cell as estimated by 80L5C4 IgG binding (31). The amount of gp80 decreases steadily in the post-aggregation stages (25, 26, 31). This is reflected by a substantial decrease in the binding of gp80-Covaspheres to 18-h cells. After the formation of the tight mound, cells are apparently held together by other adhesion molecules. At least two glycoproteins of  $M_r$  95,000 and 150,000 have been implicated in mediating the EDTA-resistant type of contact sites in the second half of the developmental cycle (12, 18, 29, 31, 38). The precise functions of these adhesion molecules in morphogenesis and their relationship with one another and with the EDTA-sensitive contact sites remain to be elucidated. Studies on gp80 should provide a prototype for further analyses of these molecules.

The biased distribution of gp80-Covaspheres on aggregation-stage cells corresponds closely to the topographic distribution of gp80 on the cell surface (4). Our previous studies show that gp80 is enriched on the filopodia as well as in the polar contact zones of migrating cells. The plasma membrane is turning over rapidly during the chemotactic migration period (9) and cells are constantly withdrawing filopodia and pseudopods as well as sending out new ones at the same time (4, 36). Filopodia are especially abundant where two cells are in close apposition. They often make direct contacts with the plasma membrane or intertwine with filopodia from neighboring cells. These dynamic surface structures are probably sent out as "feelers" to explore the external environment and are involved in the initial stages of cell-cell recognition, leading to the formation of stable contacts via the adhesion molecules. It is of interest to note that the filopodia of neuronal growth cones have been shown to be important in pathfinding and adhesive interactions (19, 40). Significant amounts of N-CAM are also found associated with the lamellar and filopodial processes extending from the tip of the axon, suggesting that it may play a role in axonal guidance (41).

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