Cell Body and Flagellar Agglutinins in *Chlamydomonas reinhardtii*: The Cell Body Plasma Membrane Is a Reservoir for Agglutinins whose Migration to the Flagella Is Regulated by a Functional Barrier

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Abstract. Fertilization in *Chlamydomonas reinhardtii* is initiated when gametes of opposite mating types adhere to each other via adhesion molecules (agglutinins) on their flagella. Adhesion leads to loss of active agglutinins from the flagella and recruitment of new agglutinins from a pool associated with the cell body. We have been interested in determining the precise cellular location of the pool and learning more about the relationship between agglutinins in the two domains. In the studies reported here we describe methods for purification of mt^+ cell body agglutinins by use of ammonium sulfate precipitation, chromatography (molecular sieve, ion exchange, and hydrophobic interaction), and sucrose gradient centrifugation. About 90% of the total agglutinins were associated with the cell body and the remainder were on the flagella. Cell body agglutinins were indistinguishable from mt^+ flagellar agglutinins by SDS-PAGE, elution properties on a hydrophobic interaction column, and in sedimentation properties on sucrose gradients. The nonadhesiveness of cell bodies suggested that the cell body agglutinins would be intracellular, but our results are not consistent with this interpretation. We have demonstrated that brief trypsin treatment of deflagellated gametes destroyed all of the cell body agglutinins and, in addition, we showed that the cell body agglutinins were accessible to surface iodination. These results indicated that *C. reinhardtii* agglutinins have a novel cellular disposition: active agglutinins, representing ~10% of the total cellular agglutinins, are found only on the flagella, whereas the remaining 90% of these molecules are on the external surface of the cell body plasma membrane in a nonfunctional form. This segregation of cell adhesion molecules into distinct membrane domains before gametic interactions has been demonstrated in sperm of multicellular organisms and may be a common mechanism for sequestering these critical molecules until gametes are activated for fusion. In experiments in which surface-iodinated cell bodies were permitted to regenerate new flagella, we found that the agglutinins (as well as the 350,000 M_r, major flagellar membrane protein) on the newly regenerated flagella were iodinated. These results indicate that proteins destined for the flagella can reside on the external surface of the cell body plasma membrane and are recruited onto newly forming flagella as well as onto preexisting flagella during fertilization.

DURING fertilization in the biflagellated alga *Chlamydomonas reinhardtii*, gametes of opposite mating types (mt^+ and mt^-) adhere to each other via agglutinin molecules on the surfaces of their flagella. This adhesive interaction induces a cAMP-mediated signal (Pijst, et al., 1984; Pasquale and Goodenough, 1987) leading to several events in fertilization including (*a*) secretion of a serine protease that converts an inactive prometalloprotease to an active enzyme, g-lysin, (Buchanan et al., 1989; Snell et al., 1989; Adair and Snell, 1990) that releases the cell wall (*b*) erection of an actin-filled fertilization tubule (Friedmann et al., 1968; Detmers et al., 1983); and (*c*) fusion of the gametes to form a zygote. In addition to generating this signal, interaction between complementary agglutinins also leads to their rapid inactivation (Snell and Roseman, 1979). Using an impotent mt^+ mutant (*imp*-1) that can agglutinate but not fuse we identified a pool of agglutinin molecules that replaces agglutinins as they are lost from the flagella during adhesion (Snell and Moore, 1980). Saito et al. (1985) confirmed these results and found that the gametic cell body contained >90% of the total cellular agglutinins. While this suggested that the cell body contains the pool of agglutinins that we had identified in our earlier experiments, it left open the question of whether the cell body agglutinins were intracellular or on the cell surface.

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The lack of adhesiveness of cell bodies led Saito et al. (1985) to conclude that cell body agglutinins were intracellular. Although these workers also showed that cell body agglutinins were removed from cells by trypsin treatment of intact gametes, they suggested that as the agglutinins were stripped from the flagella by these agents, they were replaced by adhesion molecules from within the cell body until all of the cell body agglutinins also were depleted. More recently, Goodenough (1989) noted that agglutinins previously shown to be present on flagella (Goodenough et al., 1985) were not detectable on the outer surface of cell body plasma membrane using quick-freeze, deep-etch EM. Because replacement of lost flagellar agglutinins is such a rapid and important cellular event, we became interested both in more clearly identifying the location of the cell body agglutinins and in learning more about the relationship between the cell body and flagellar forms of these molecules.

Interest in the location of the cell body agglutinins also derives from studies on the organism's ability to regenerate new flagella after loss of the original pair. Rosenbaum et al. (1969) have shown that cells contain a pool of flagellar molecules used during flagellar regeneration. Although the internal constituents (e.g., tubulin) of the regenerated flagella are likely of cytoplasmic origin, the membrane components of regenerating flagella could be derived either from intracellular vesicles or the plasma membrane of the cell body.

In the studies reported here we describe a method for the purification of cell body agglutinins and demonstrate that they are located on the outer surface of the cell body plasma membrane. We also describe methods for depleting flagellar agglutinins without inducing sexual signaling. Surprisingly, under these conditions the cell body agglutinins are unaffected. This indicates that in the absence of a sexual signal, preexisting cell body agglutinins are unavailable for utilization on flagella. Finally, we show that during flagellar regeneration the newly formed flagella acquire agglutinins from this pool of cell surface agglutinins.

Materials and Methods

Materials

Trizma(Trisbase),cycloheximide,(3-[2(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl] glutarimide), papaverine (1-[(3,4-dimethoxyphenyl) methyl]-6,7-dimethoxyisoquinoline), dibutyryl cAMP, Pipes, EDTA, BSA, apoferritin (horse), thyroglobulin (bovine), myoglobin (horse), ovalbumin, soybean trypsin inhibitor, lactoperoxidase, PMSF, and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO); ^{125}I was from Amersham Corp. (Arlington Heights, IL); Hepes was from Research Organics Inc. (Cleveland, OH). DMSO was from J.T. Baker Chemical Co. (Phillipsburg, NJ). All other chemicals were reagent grade. Cycloheximide (CH) was used at 10 μg/ml. Stock solution of dibutyryl cAMP was 50 mM dibutyryl cAMP in 10 mM Pipes, pH 7.2. Stock solution of papaverine was 10 mM in DMSO and was prepared by heating the mixture to 60°C until the papaverine went into solution.

Cells and Cell Cultures

Chlamydomonas reinhardtii strains (available from the *Chlamydomonas* Genetics Center, Duke University) 21*gr* (*mt*$^+$) [CC-1690], 6145*c* (*mt*$^-$) [CC-1691] and the nonfusing mutant, *imp*-1 (*mt*$^+$) [CC-462], were cultured at 25°C in medium I or medium II (medium I supplemented with 3 g/liter of sodium acetate trihydrate and three times the amount of the phosphate buffers) (Sager and Granick, 1954; Harris, 1989) on a 12-h light/dark cycle as previously described (Kates and Jones, 1964). Gametic cells were obtained as previously described (Snell, 1976*a*) by transferring vegetative cells (4–7 × 10^6 cells/ml) 6 h after the beginning of the light period into nitrogen-free (N-free) medium (Sager and Granick, 1954) modified to contain 0.15 g/liter of KH_2PO_4 and 0.3 g/liter of K_2HPO_4.

Cells were counted on a hemocytometer or with a Coulter counter with the window set to detect >95% of all the single cells, as previously described (Snell and Roseman, 1979).

Isolation of Cell Bodies and Flagella

Flagella were separated from cell bodies by a modification of the pH-shock method of Witman et al. (1972). Whole cells were suspended in N-free medium or Hepes-Calcium buffer (HC; 10 mM Hepes, 1 mM $CaCl_2$, pH 7.2). The pH of a vigorously stirred suspension of cells was rapidly lowered to 4.2 by the dropwise addition of 0.5 M acetic acid. The sample was held at this pH for 1 min, examined microscopically to ensure that deflagellation had occurred, and then the pH rapidly was raised to 7.2–7.4 by the dropwise addition of 0.5 M KOH. To harvest the cell bodies, the suspension was centrifuged at 3,000 rpm (1800 g_{av}) for 3 min at 4°C in a conical polystyrene centrifuge tube (rotor 253; International Equipment Co., Needham Heights, MA). The supernatant, containing the flagella, was removed by aspiration and centrifuged at 16,000 rpm (30,000 g_{av}) for 20 min at 4°C in a rotor (model SA600; Sorvall Div., DuPont Corp., Newton, CT) to harvest the flagella. The sedimented flagella were resuspended in 10 mM Tris buffer, pH 7.2, at 4°C (typically 0.5 ml for 5 × 10^8 cells), frozen immediately in liquid nitrogen and stored at −80°C until processed to determine the agglutinin levels as described below. The sedimented cell bodies were resuspended to twice their sedimented volume in 10 mM Tris buffer, pH 7.2, at 4°C, frozen immediately in liquid nitrogen and stored at −80°C until processed.

Solubilization of Agglutinins

Cell body or flagellar samples were thawed rapidly in a 37°C water bath and placed immediately on ice. The cell body samples were disrupted either by passing them through a French pressure cell (1 or 3/8 in. diameter pressure cells; American Instruments Co., Silver Springs, MD) at pressures of 8,000–10,000 pounds/in.^{2} (Saito et al., 1985), or by exposure to two 10-s ultrasonic bursts separated by a 1-min rest using setting no. 7 on a sonicator (model W185-F; Heat Systems-Ultrasonic, Inc., Farmingdale, NY) fitted with a step microtip. In both cases, suspensions were checked microscopically to ensure total cellular disruption. Flagellar samples were disrupted by sonicating them once for 10 s. In experiments not shown, we determined that the amount of agglutinin released by sonication was equivalent to or slightly greater than the amount released after detergent (0.5% Triton X-100 or 0.5% Triton X-114) or French pressure cell disruption of the gametes. The cell bodies disrupted by the French pressure cell were used for the purification of the cell body agglutinins. Immediately after these cells were disrupted, EDTA and PMSF were added to the samples to produce final concentrations of 4.0 and 1.0 mM, respectively. These samples were then centrifuged at 43,000 rpm (145,000 g_{av}) for 2 h at 4°C in a rotor (Ti-45) using an ultracentrifuge (Beckman Instruments, Fullerton, CA). In experiments with smaller volumes of cells an ultracentrifuge (model TL-100; Beckman Instruments) was used to clarify the disrupted cell body or flagellar suspensions by centrifuging at 65,000 rpm (150,500 g_{av}) for 25 min at 4°C using the 100.2 rotor.

Protein Determination

Protein levels were determined with the BCA (bicinchoninic acid) method of Pierce Chemical Co. (Rockford, IL) using crystalline BSA as the standard.

In Vitro Assay for Agglutinin Activity

A modified version of the dried-spot bioassay of Adair et al. (1982) was used to assay the agglutinin levels of cell body and flagellar extracts. Briefly, samples were serially diluted in distilled water and 2-μl samples were dried onto a clean glass microscope slide. The slide was rinsed briefly with distilled water and 2–3 ml of *mt*$^-$ gametes in N-free medium were applied to the slide. After 3 min the agglutinin activity of the sample was determined visually by noting the most dilute sample to which the gametes were able to adhere. The reciprocal of this dilution multiplied by the original volume of the sample in ml represents the agglutinin activity for that sample (agglutinin units). Typically we obtained ~2 agglutinin units/10^6 cells with 10% of the agglutinin activity associated with the flagella and 90% of the agglutinin activity associated with the cell bodies.

Aggregation Assay

An electronic particle counter (model ZBI; Coulter Electronics; Hialeah, FL) fitted with an electrode containing a 100-μm bore aperture was used to quantitatively determine cell concentration and to assay cell aggregation, as previously described (Snell and Roseman, 1979). Briefly, threshold settings were adjusted for each experiment to detect at least 75% of the single cells. Typical settings were 1/aperture current = 0.5, 1/amplification = 0.354. Threshold settings were 15–20 for the lower and 70–80 for the upper. For counting, cells were diluted 1:100 or 1:500 in ice-cold or room temperature medium II to yield an initial Coulter count of 20,000 to 25,000. Cell aggregates fall outside the upper threshold setting and are not detected.

Ammonium Sulfate Precipitation

A 100% saturated, 4°C solution of ammonium sulfate (AS)[1] was added dropwise to the sample, while it stirred in an ice-water bath, until the desired final percent AS saturation was achieved. The sample was stirred for at least 15 min to allow maximal protein precipitation. The material precipitated at 45% saturation was discarded while the material precipitated at 55% saturation was harvested. For agglutinin purification the 55% precipitated material was centrifuged at 12,000 rpm (20,000 g_{av}) for 30 min at 4°C in a GSA rotor, resuspended in 10 mM Tris, pH 7.2, and concentrated and dialyzed in a Micro ProDiCon model MPDC-20 dialysis system (Bio-Molecular Dynamics, Beaverton, OR).

PAGE

SDS-PAGE was performed on 15 × 13 × (0.075 or 0.15 cm) slab gels containing a gradient of 2–16% acrylamide and 3–8 M urea with a 2% Laemmli (1970) stacking gel. In some experiments a 2.2–20% acrylamide gradient gel was used (Homan et al., 1987) without a stacking gel. Walls between lanes were made by dispensing 1% warm agarose through a syringe. Gels were run at constant current until the tracking dye reached the bottom of the gel. Gels were fixed and incubated with periodate (Dubray and Bezard, 1982) before silver staining (Merrill et al., 1981). High molecular weight standard protein markers were from Bio-Rad Laboratories (Richmond, CA).

Chromatography

Molecular Sieve. After dialyzing and concentrating to 1–2 ml in 10 mM Tris, pH 7.2, the 55% ammonium sulfate cuts of cell body extracts were loaded onto a Bio-Gel A-15M (Bio-Rad Laboratories, Richmond, CA) 1.5 × 65 cm column equilibrated with 10 mM Tris, pH 7.2 and fractionated at 4°C. Fractions (1.25 ml) were collected in polyethylene tubes.

HPLC: Hydrophobic Interaction Chromatography for Agglutinin Purification. Material that bound to a TSK phenyl 5PW 7.5 × 75 mm column with guard column (Beckman Instruments), equilibrated with 1.0 M AS in 0.1 M K_2PO_4, pH 7.0 (Buffer A), was eluted using HPLC (Waters Instruments, Inc., Rochester, MN) according to the following protocol using a flow rate of 1 ml/min (buffer B; 0.1 M K_2PO_4, pH 7.0): 0–5 min, 100% buffer A; 5–15 min, linear gradient to 60% buffer B; 15–25 min, 60% buffer B; 25–35 min, linear gradient to 100% buffer B; 35–50 min, 100% buffer B.

Velocity Sedimentation

To determine sedimentation coefficients, samples were loaded onto linear 5–20% sucrose gradients prepared according to the method described in Beckman products application bulletin DS-640A for the tabletop ultracentrifuge (TL-100). Samples were centrifuged in rotor TLS-55 at 55,000 rpm (200,000 g_{av}) for 3.75 h at 4°C. Fractions (100 μl) were collected from above and analyzed as described in Results.

Cell Wall Removal and Wall Loss Assay

To remove the cell walls, mt^+ gametes (2.5 × 10^7 cells/ml in HC buffer) were incubated in a 1:5 dilution of crude g-lysin prepared as previously described (Buchanan et al., 1989). After bubbling 15 min under fluorescent lights, the sample was assessed for cell wall loss. To do this, 50 μl of the sample was mixed with 500 μl of a detergent solution (0.075% Triton X-100 in 10 mM EDTA), vortexed, centrifuged with a model B microfuge (Beckman Instruments) for 10 s and the OD_{435} of the supernatant determined to quantify the amount of released chlorophyll. The quantity of chlorophyll detected directly reflects the number of cells that have undergone cell wall loss (Snell, 1982).

Iodination

De-walled, deflagellated impotent mt^+ (*imp*-1) gametes at 2 × 10^9 cells in 4 ml of 4°C HC buffer were iodinated vectorially by the lactoperoxidase/hydrogen peroxide method of cell surface labeling (Marchalonis, 1969). 2 mCi of ^{125}I were mixed with the cells and the solution placed in an ice-water bath. Lactoperoxidase (1 mg/ml in HC) and hydrogen peroxide (diluted 1:1,000 in HC) were added to the cell-iodide solution by the following protocol: 50 μl of lactoperoxidase was added at t=0, 4, and 8 min; 10 μl of hydrogen peroxide was added at t=0, 2, 4, 6, 8, and 10 min, gently mixing the cell after each addition. At t=12 min, the iodination was terminated by adding 35 ml of 4°C HC. The suspension was centrifuged at 3,000 rpm (1400 g_{av}) for 5 min at 25°C in a tabletop centrifuge (rotor 958; Damon/IEC) and the supernatant removed. Cells were washed five times by resuspending them in 35 ml of 4°C N-free medium followed by centrifugation as above. After the fifth wash, the cells were resuspended in 70 ml of 25°C N-free medium, split into two 35-ml samples, and allowed to regenerate their flagella while being gently aerated under fluorescent lights. After the cells had regenerated their flagella each sample was washed once more as above. Examination of the suspension after these treatments showed that >95% of the cells were fully flagellated and motile. In the experiments to deplete gametes of their labeled cell body agglutinins, cells were mated with unlabeled mt^- gametes. One of the above 35-ml samples of labeled *imp*-1 mt^+ cells was mixed with an equal number of unlabeled mt^- gametes and allowed to agglutinate for 45–60 min to diminish the pool of labeled mt^+ cell body agglutinins (see Fig. 10 *B*). As a control the other 35-ml sample was mixed with an equal number of unlabeled *imp*-1 mt^+ gametes for the same length of time.

Autoradiography

Labeled cell body and flagellar fractions were analyzed by SDS-PAGE and autoradiography. Gels were dried between two sheets of cellophane (Hoefer Scientific Instruments, San Francisco, CA), placed on preflashed Kodak XAR film in a cassette with two intensifying screens (Cronex Lightningplus, DuPont Corp.) placed at −80°C.

mAb

Production and characterization of the mAb 2b40, which blocks adhesion of mt^+ gametes, was previously described (Snell et al., 1986). Ascites fluids were prepared from pristane-primed mice injected intraperitoneally with mAb 2b40 hybridoma cells. An mAb against a microtubule-associated protein, 1B1, provided by Dr. George Bloom (University of Texas Southwestern Medical Center, Dallas) (Bloom et al., 1985) was used as an irrelevant antibody.

Results

Purification of Cell Body Agglutinins

Cell body agglutinins were obtained from deflagellated gametes by use of ammonium sulfate precipitation of cell homogenates as described in Materials and Methods. After molecular sieve and ion exchange chromatography we achieved >180-fold purification with 5–50% recovery of activity. As will be shown below, these methods yielded a very high M_r molecule that was indistinguishable from flagellar agglutinin. Fig. 1 shows information obtained from molecular sieve chromatography of the ammonium sulfate fraction. (*Top*) The profile of agglutinin activity determined by the dried-spot bioassay (see Materials and Methods); (*bottom*) shows the corresponding fractions analyzed on 2–16% SDS-PAGE gradient gels. Electrophoresis revealed a high molecular weight band ~1 cm from the top of the gel (*arrow*) that was coincident with agglutinin activity. This electrophoretic behavior is similar to that of flagellar agglutinins (data not shown; and Adair et al., 1982).

Dialyzed active fractions from the molecular sieve col-

1. *Abbreviation used in this paper:* AS, ammonium sulfate.

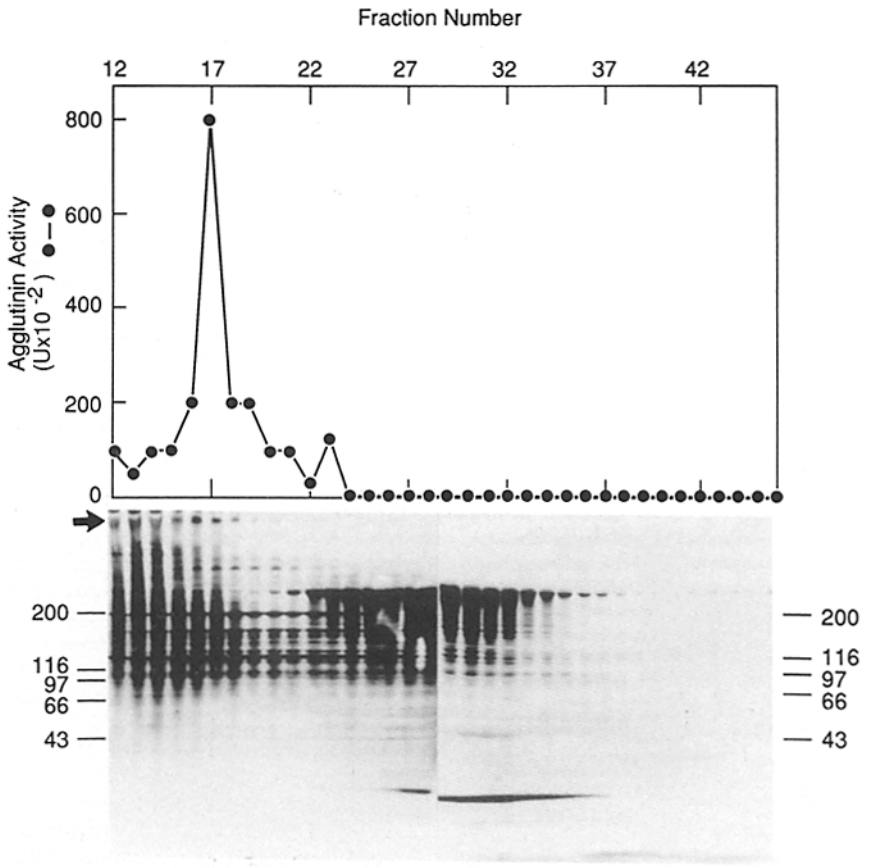

Figure 1. Molecular sieve chromatography of cell body agglutinin on BioGel A-15M. Partially purified cell body agglutinin extract from ~5 × 10^{11} mt^+ gametes was loaded onto a BioGel A-15M gel filtration column as described in Materials and Methods. (*Top*) Agglutinin activity (*closed circles*) of the indicated fractions off the column; and (*bottom*) shows the 2–16% SDS-PAGE profile of the same fractions. Polypeptides were visualized by silver staining. *Arrow*, position of the high M_r polypeptide that copurified with agglutinin activity. The numbers along the side of the gel represent the M_r of the molecular weight standards.

umn, which were enriched 66-fold over the starting material, were applied to an HPLC hydrophobic interaction column (TSK phenyl 5PW; Beckman Instruments). Fig. 2 shows that agglutinin activity was separated from the majority of proteins (OD_{280} profile, *top*) and the high M_r band (*arrow, bottom*) again was coincident with agglutinin activity (*top*).

To investigate the physical properties of cell body agglutinins and for further fractionation, peak fractions of agglutinin activity from ion exchange chromatography were analyzed on 5–20% sucrose density gradients. The results in Fig. 3 show that the agglutinin activity (*top*) again copurified with the high M_r polypeptide (*arrow, bottom*). Comparison of the sedimentation of the agglutinin to the sedimentation of known proteins showed that the sedimentation coefficient of the cell body agglutinin was 12.3S (data not shown), equivalent to the 12S value reported by Adair et al. (1982) for the flagellar agglutinin. In addition, cell body and flagellar agglutinins were indistinguishable by HPLC hydrophobic interaction chromatography (not shown).

To confirm that the high M_r molecule was the agglutinin, a curtain gel of the active fractions pooled from a molecular sieve column was run on SDS-PAGE and slices were extracted and assayed for agglutinin activity. Only the portion of the gel containing the high M_r polypeptide had activity (data not shown).

Cell Body Agglutinins Are on the External Surface of the Plasma Membrane in an Inactive Form

That cell bodies do not participate in the adhesive interaction between mt^+ and mt^- gametes indicates either that cell body agglutinins are internal or that they are on the surface, but not functional. Although we (data not shown) and Saito et al. (1985) have shown that both cell body and flagellar agglutinins are removed by trypsinization of flagellated gametes with walls, it was possible in those experiments that only flagellar agglutinins were sensitive to trypsin. Saito et al. (1985) suggested that as agglutinins were lost from the flagella by trypsinization they might have been replaced by agglutinins from the cell body. To learn more about the location of cell body agglutinins on cells without flagella, de-walled gametes whose flagella were removed were incubated with 0.05% trypsin for 10 min in an ice-water bath. Soybean trypsin inhibitor was added at the end of the incubation and the cells were washed by centrifugation. As controls soybean trypsin inhibitor and trypsin were added together at the start of the incubation to one sample, whereas another sample was neither de-walled nor trypsin-treated. After the treatments, the samples were assayed for agglutinins in the dried-spot bioassay. The results in Table I show that removal of the cell wall did not affect the levels of cell body agglutinins; more importantly, all of the cell body agglutinins detectable in the assay were destroyed by the trypsinization, indicating that they were located on the outer surface of the cell body plasma membrane.

Although these experiments indicated that the cell body agglutinins detectable in the in vitro dried-spot bioassay were on the external surface of the cell, it was possible that there were internal cell body agglutinin precursors that had no agglutinin activity in this assay. Putative precursors might serve as another reservoir for cell body and flagellar agglutinins and would acquire activity in the dried-spot bioassay

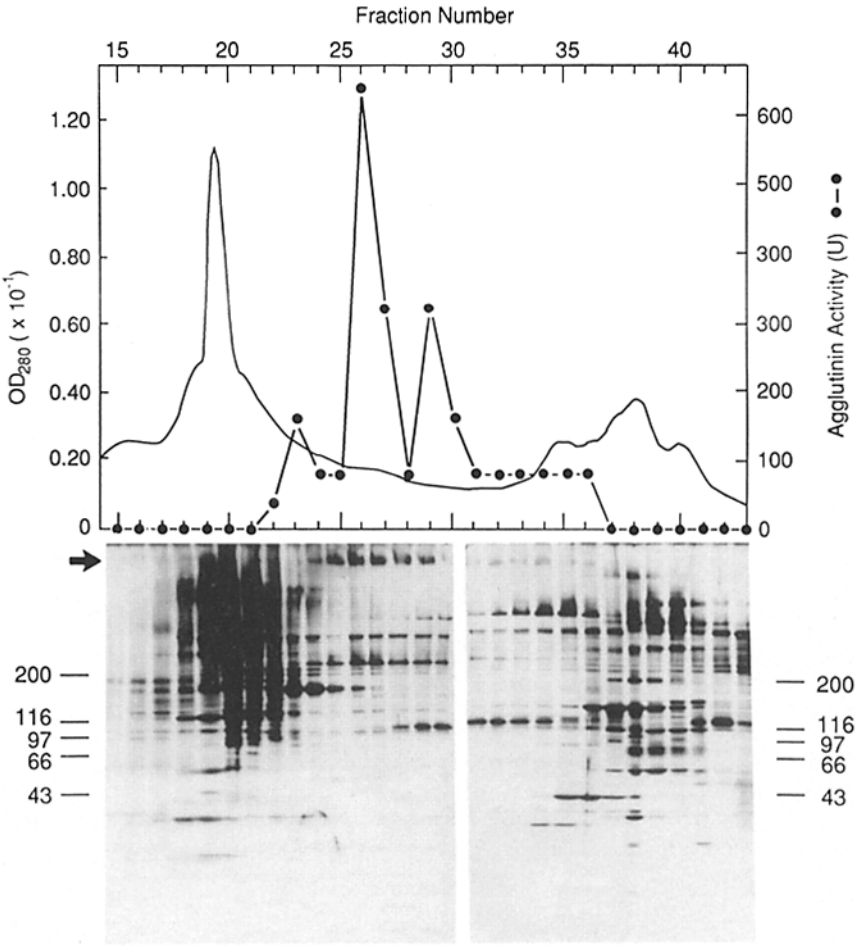

Figure 2. Hydrophobic interaction chromatography of partially purified cell body agglutinin. Fractions off the A-15M column with peak agglutinin activity were pooled, dialyzed, brought to 1.0 M AS, and loaded onto an HPLC (Waters Instruments, Inc.) TSK phenyl 5PW column as described in Results. The column was eluted as described in Materials and Methods and the OD_{280} was monitored. (*Top*) shows the OD_{280} profile (*solid line*) and the agglutinin activity (*closed circles*), determined after dialysis into 10 mM Tris, pH 7.2, of the indicated fractions. (*Bottom*) 2–16% SDS-PAGE profile of the same fractions. Polypeptides were visualized by silver staining. *Arrow*, Position of the high molecular weight polypeptide that copurified with agglutinin activity. The numbers along the side of the gel represent the M_r of the molecular weight standards.

only after being delivered to the cell body of flagellar surface. When we tested this by inducing sexual signaling in de-walled, flagellated, trypsinized gametes by incubating them in dibutyryl cAMP and papaverine (a phosphodiesterase inhibitor), we found that gametes did not become agglutinable. Pasquale and Goodenough (1987) and Goodenough (1989) have shown that similar treatment of nontrypsinized gametes induces sexual signaling and movement of agglutinins from the cell body to the flagella, results that we have confirmed (see below). While these results cannot rule out the possibility of inactive, intracellular agglutinins, if there is an internal pool of agglutinin precursors not detectable in the dried-spot bioassay, it is not induced to move and become active under these conditions.

Table I. Effects of Wall Removal and Trypsin on Cell Body Agglutinins

mt^+ gametes	Treatment	Cell body agglutinin units
Intact	None	2,560
De-walled	None	2,560
	Trypsin	0
	Trypsin plus inhibitor	2,560

mt^+ gametes (~5 × 10^8/5 ml in polystyrene tubes) were deflagellated and placed in an ice bath. Cells were treated with 0.05% trypsin for 10 min and then 0.1% soybean trypsin inhibitor was added. The de-walled samples were previously treated with g-lysin to remove the walls as described in Materials and Methods. In the trypsin plus inhibitor sample soybean trypsin inhibitor and trypsin were added simultaneously. The cells were washed twice by bringing the volume up to 35 ml with N-free medium and sedimenting the cells by centrifugation. Sedimented cells from the last centrifugation were resuspended in 10 mM Tris, pH 7.2 to 0.5 ml, sonicated, and assayed for agglutinin levels with the in vitro dried-spot bioassay.

Flagellar Agglutinins Can Be Depleted without Inducing a Sexual Signal

We next wanted to learn more about the relationship between these two cell surface domains. We and others have shown that there is a pool of agglutinins that moves to the flagella during sexual signaling (Snell and Roseman, 1979; Snell and Moore, 1980; Saito et al., 1985; Snell et al., 1986; Goodenough, 1989; Hunnicutt, 1989) but we wanted to determine if preexisting cell body agglutinins were accessible to the flagella in the absence of a sexual signal. To ask this question we developed methods for depleting flagella agglutinins without inducing sexual signaling by use of an adhesion-blocking mAb, mAb 2b40, that was made against enriched fractions of mt^+ flagellar agglutinins (Snell et al., 1986). Previously, we showed that when mt^+ gametes were incubated with soluble mAb 2b40 and then mixed with mt^- gametes agglutination was blocked; moreover, mt^+ gametes (but neither mt^- gametes nor vegetative cells of either mating type) bound to Sepharose beads derivatized with mAb 2b40.

In our previous studies with mt^+ gametes adhering to

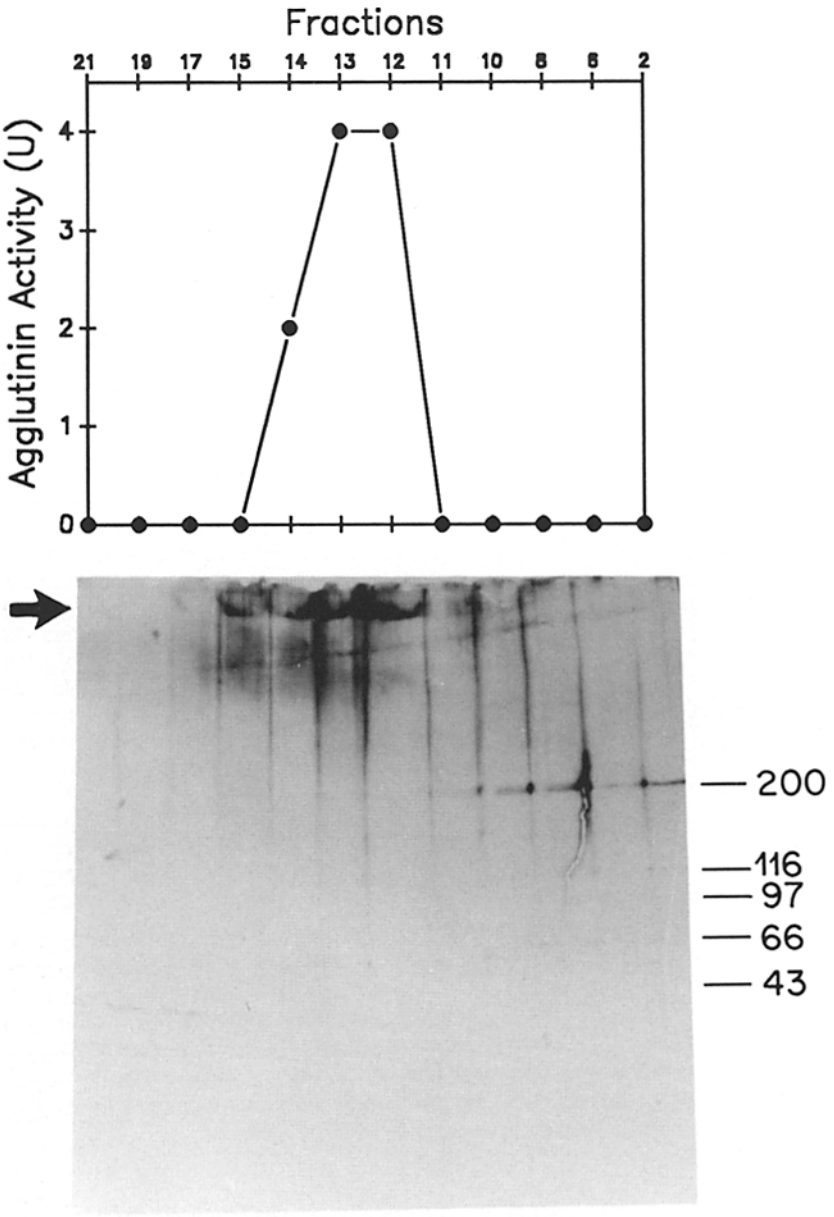

Figure 3. Sedimentation of cell body agglutinin on a 5–20% sucrose gradient. Cell body agglutinin (0.1 ml in 10 mM Tris, pH 7.0) partially purified by molecular sieve and ion exchange chromatography was fractionated on a 2.0-ml linear sucrose gradient by centrifugation. 100-μl fractions were collected from the top and assayed, without dialysis, for agglutinin activity. (*Top*) Agglutinin activity (*closed circles*) for the indicated fractions; (*bottom*) 2.2–20% SDS-PAGE profile of the same fractions. Polypeptides were visualized by silver staining. *Arrow*, position of the high molecular weight polypeptide that co-purified with agglutinin activity. The numbers along the side of the gel represent the M_r of the molecular weight standards.

mAb 2b40-derivatized Sepharose beads we showed that the agglutinins were released from the flagella and could be recovered on the beads (Snell et al., 1986). Although the molecular mechanism is unknown, this is similar to the loss of agglutinins that occurs when gametes of opposite mating types adhere to one another (Snell and Moore, 1980; Saito et al., 1985) and is consistent with the idea that the antibody blocks adhesion by inducing release of the agglutinins from the flagella. In our earlier studies we also showed that cells that bound to the mAb 2b40-derivatized beads underwent cell wall loss, indicating that sexual signaling was induced (Snell et al., 1986). When we incubated mt^+ gametes with soluble mAb 2b40 (concentrations from 0.1 to 1000 μg/ml ascites fluid/ml), however, there was no wall loss, indicating that the soluble antibody did not cause sexual signaling (data not shown).

Having established that the antibody blocked adhesion without inducing a sexual signal, we next wanted to determine if the effects of the antibody on flagellar adhesiveness were reversible. To do this, gametes were incubated with mAb 2b40 for 180 min. At this time the Coulter counter assay showed that aggregation was inhibited 100% (Fig. 4 *A*, *hatched box*). Gametes then were washed out of the mAb 2b40 and resuspended in fresh medium. 80 and 170 min after the antibody had been washed away, the cells were tested again for their ability to aggregate with fresh mt^- gametes. The results shown in Fig. 4 *A* indicate that the antibody-induced loss of flagellar adhesiveness was reversible if the cells were washed out of the antibody. Nearly 75% of the initial level of adhesiveness was re-established within 80 min after resuspension in fresh medium (*solid box*) with a small increase at 170 min after resuspension (*stippled box*).

Protein Synthesis Is Required to Reexpress Flagellar Agglutinins after Antibody-induced Depletion

To ascertain if recovery of adhesiveness after antibody-

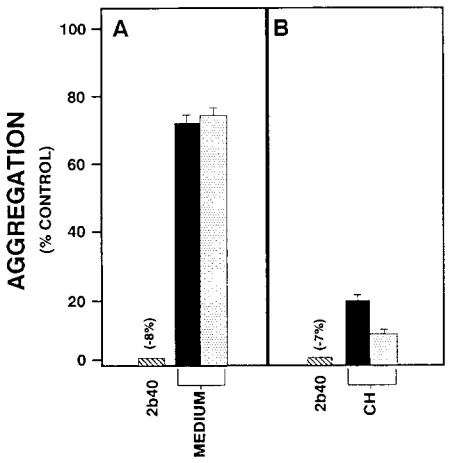

Figure 4. The effects of CH on recovery of adhesiveness after treatment of mt^+ gametes with mAb 2b40. mt^+ gametes (10^7 cells/ml) were incubated with mAb 2b40 (170 μg/ml ascites fluid) in polystyrene tubes and gently agitated to keep the cells in suspension. After 180 min a portion of the cells was mixed with an equal number of untreated mt^- gametes and their agglutinability assessed with the Coulter counter assay (*hatched boxes*). The remaining cells were washed twice by centrifugation (2,250 *g* for 5 min at 4°C; IEC rotor 253) and resuspended in N-free medium with (*A*) or without (*B*) CH. 80 (*solid boxes*) and 170 (*stippled boxes*) min after resuspension samples again were tested for agglutinability. The results are expressed as the percent aggregation relative to untreated control cells. For these experiments the control cells averaged 76% aggregation. In this and subsequent figures, error bars represent the standard deviations of the mean from at least duplicate samples.

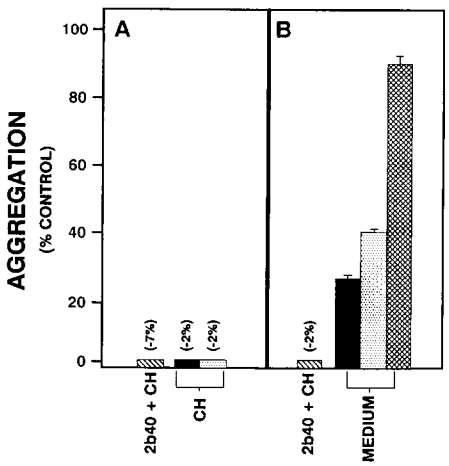

Figure 5. The effects of CH on recovery of adhesiveness after treatment of mt^+ gametes with mAb 2b40 and CH. mt^+ cells (10^7 cells/ml) were incubated with mAb 2b40 as described in the legend to Fig. 4 but in the presence of CH and the agglutinability was determined (*hatched boxes*) using the Coulter counter assay. After 180 min, the cells were washed twice by centrifugation and resuspended in N-free medium with (*A*) or without (*B*) CH. Agglutinability of the gametes was assessed 80 (*solid boxes*), 170 (*stippled boxes*), and (in *B*) 300 (*crosshatched box*) min after the cells were resuspended. The results are expressed as the percent agglutinability relative to untreated control cells. For these experiments the control cells averaged 76% aggregation.

induced depletion of agglutinins required protein synthesis, we repeated the experiment in the presence of the protein synthesis inhibitor cycloheximide. Gametes mt^+ were incubated with mAb 2b40 (Fig. 4 *B, hatched box*) and then washed out of the antibody into medium containing cycloheximide (CH; 10 μg/ml). As shown in Fig. 4 *B*, CH greatly inhibited recovery. Adhesiveness reached only 20% of the control levels after 80 min (*solid box*) and was 10% of the control at 170 min (*stippled box*). Moreover, if CH was included both during the initial incubation of gametes with mAb 2b40 as well as in the medium the cells were washed into after the antibody was removed, recovery of agglutinability was completely inhibited. Fig. 5 *A* shows that, in the presence of CH, immediately before the cells were washed out of mAb 2b40 (*hatched box*), as well as 80 (*solid box*) and 170 min (*stippled box*) after antibody removal the cells were completely nonadhesive, even though they were fully viable and motile.

This inhibition by CH was reversible if cells initially incubated with mAb 2b40 and CH (Fig. 5 *B, hatched box*) were washed into fresh medium. Fig. 5 *B* demonstrates this recovery of agglutinability. 80 (*solid box*) and 170 min (*stippled box*) after washing, the cells had recovered ~30 and 40%, of the control level of aggregation, respectively; and >95% recovery was noted after 300 min (*cross-hatched box*). An irrelevant mAb appeared to have no effect on the gametes' agglutinability that could not be accounted for by CH alone under identical incubation conditions (not shown). Together, these results demonstrate the existence of a biosynthetic pathway for flagellar agglutinins. In addition, they show that if flagellar agglutinins are removed, in the absence of a sexual signal, proteins synthesis is required for their replacement.

Cell Body Agglutinins Are Unaffected under Conditions that Completely Deplete Flagellar Agglutinins

Having established a method for depleting flagellar agglutinins without inducing sexual signaling, we could determine the effects of these conditions on cell body agglutinins. To do this, mt^+ gametes were incubated with mAb 2b40 and CH for 180 min at which time the Coulter counter assay showed that the mt^+ cells had completely lost their adhesiveness. The gametes were then harvested, deflagellated, and the cell body and flagellar fractions assayed for agglutinin levels using the dried-spot bioassay. As expected, flagellar agglutinins were not detectable (Fig. 6, *hatched box*) compared with control, untreated mt^+ gametes.

Surprisingly, the cell bodies from the mAb 2b40-treated gametes (Fig. 6, *solid box*) showed the same amount of agglutinins as the nontreated controls. These results showed that agglutinins in the two domains (cell body and flagella) could be manipulated independently. Furthermore, the results indicated that a functional barrier prevented utilization of preexisting cell body agglutinins by the flagella, since simply removing the flagellar agglutinins did not result in their replacement from the cell body.

Dibutyryl cAMP and Papaverine Cause Cell Body Agglutinins to Move onto the Flagella

Having presented evidence for a functional barrier between the cell body and the flagella we next wanted to determine whether the barrier could be overcome if we induced sexual signaling by the addition of dibutyryl cAMP and a phosphodiesterase inhibitor (Pasquale and Goodenough, 1987). To do this mt^+ gametes were depleted of their flagellar agglutinins by incubation in mAb 2b40 and CH for 180 min

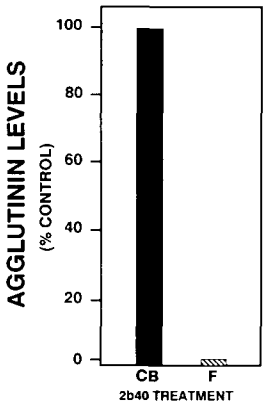

Figure 6. Levels of mt^+ cell body and flagellar agglutinins after mAb 2b40 and CH treatment. Agglutinin levels were determined using the dried-spot bioassay on cell body (*solid box*) and flagellar (*hatched box*) fractions of gametes that had been pretreated with mAb 2b40 and CH for 180 min and then washed out of the antibody but kept in the presence of CH (*A*) as described for Fig. 5 *A*. The results are expressed as the percent relative to untreated control gametes. For this experiment control cell body and flagellar agglutinin levels were 2,560 and 320 agglutinin units, respectively.

and then washed out of the antibody but kept in the continued presence of CH. The Coulter counter assay showed that there was 95% inhibition of agglutinability under these conditions (data not shown). These non-agglutinable cells were split into two equal portions. One was incubated with dibutyryl cAMP and papaverine in CH to induce sexual signaling, whereas the other portion was incubated with CH alone. After 30 min, the gametes were tested for their adhesiveness using the Coulter counter assay and their levels of cell body and flagellar agglutinins were determined. Fig. 7 *B* shows that cells that had been induced to undergo sexual signaling (+ cAMP) reacquired the ability to aggregate as well as control cells treated with irrelevant mAb (Fig. 7 *A*). Whereas, the cells that had not been induced to signal remaining inhibited in their capacity to aggregate with untreated mt^- gametes (Fig. 7 *B*, − cAMP).

When the levels of cell body and flagellar agglutinins of these cells were quantified using the in vitro dried-spot bioassay, we found the cells that had undergone sexual signaling were devoid of cell body agglutinins (Fig. 8 *B*, *solid box*), whereas their flagella expressed normal agglutinin levels (Fig. 8 *B*, *hatched box*). Cells that had not been incubated in dibutyryl cAMP and papaverine had high levels of cell body agglutinins (Fig. 8 *A*, *solid box*) and undetectable flagellar agglutinins (*hatched box*).

To determine if the cell wall prevented the movement of agglutinins, we depleted mt^+ gametes of their flagellar agglutinins with the mAb 2b40 and CH and then treated these cells with lysin to remove their cell walls. The cells were then tested for agglutinability. Removal of the cell walls did not cause the cells to reacquire their agglutinability. If, however, we treated these cells with dibutyryl cAMP and papaverine for 30 min and then retested for agglutinability we observed complete restoration in their ability to adhere to mt^- gametes, indicating that the cell wall was not a barrier (data not shown).

Surface Iodination Labels Cell Body Agglutinins, which Can Be Incorporated onto Regenerating Flagella

As an independent method for determining if agglutinins were on the surface of the cell body and could move to the flagella we used surface iodination. To do this de-walled gametes whose flagella were removed were iodinated by the

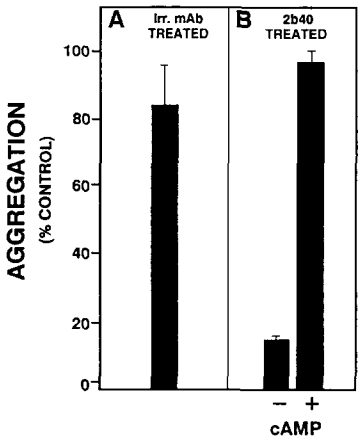

Figure 7. Effects of dibutyryl cAMP and papaverine on adhesiveness of mt^+ gametes pretreated with mAb 2b40 and CH. mt^+ gametes (10^7 cells/ml) were treated with mAb 2b40 (200 μg/ml) and CH for 180 min, washed twice by centrifugation to remove the antibody (see legend to Fig. 5), and resuspended to 5×10^7 cells/ml in HC buffer with CH. Gametes were treated with dibutyryl cAMP (10 mM in 2 mM Pipes, pH 7.2) and papaverine (0.1 mM in DMSO) for 30 min (*B*, + *cAMP*) or appropriate amounts of the buffers used for the stock solutions of the dibutyryl cAMP and papaverine (*B*, − *cAMP*) and the agglutinability of the cells was assayed using the Coulter counter. *A* shows the results for control gametes incubated with an irrelevant mAb and CH. Results are expressed as the percent aggregation relative to untreated, control cells, which was 78%.

lactoperoxidase method described in Materials and Methods, allowed to regenerate flagella, and the cell bodies and flagella then were analyzed by SDS-PAGE and autoradiography. Fig. 9 *A* (lane *1*), the autoradiograph of the cell body frac-

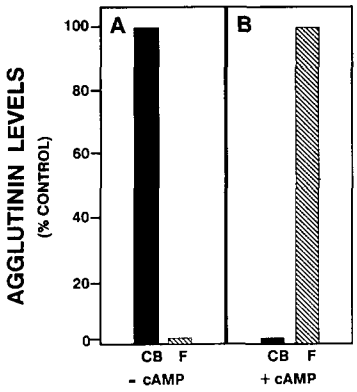

Figure 8. Effects of dibutyryl cAMP and papaverine on levels of cell body and flagellar agglutinins of gametes treated with mAb 2b40 and CH. The mt^+ gametes from Fig. 7 *B*, which initially were treated with mAb 2b40 and CH followed by treatment with dibutyryl cAMP and papaverine or control buffers, were deflagellated and the agglutinin levels of the cell bodies (*solid boxes*) and flagella (*hatched boxes*) determined using the dried-spot bioassay. *A* shows the agglutinin levels of the cells that had not been incubated with dibutyryl cAMP and papaverine and panel B shows the results for cells that had been incubated with dibutyryl cAMP and papaverine. Agglutinin levels are expressed as the percent relative to untreated, control cell bodies and flagella, which were 2,560 and 320 units, respectively.

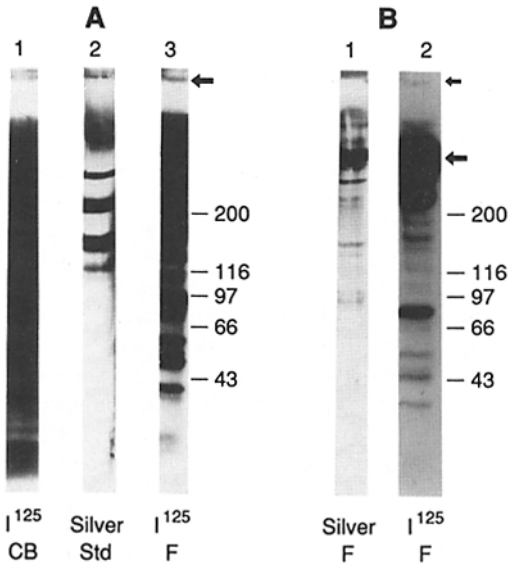

Figure 9. Iodination of cell body agglutinins by vectorial labeling and demonstration of their incorporation into regenerating flagella. (*A*) *Imp*-1 mt^+ cells were de-walled, deflagellated, and vectorially iodinated. The cells were allowed to regenerate their flagella, deflagellated, and portions of the cell body and the flagellar samples were analyzed by 2.2–20% SDS-PAGE followed by autoradiography. Lanes *1* and *3* are the autoradiographs of the cell body and flagellar fractions, respectively. Lane *2* is a silver stain of partially purified cell body agglutinin. *Arrow*, band of agglutinin. Because the agglutinins represent a small portion of the surface labeled molecules, long exposure times were used to permit the agglutinins to be visualized. (*B*) Lane *1* is the silver stain protein profile of the regenerated flagella and lane 2 is the autoradiograph of lane *1*. Lane *2* is identical to lane *3* of Fig. 9 *A* but was exposed for a shorter time to permit identification of other cell body plasma membrane proteins incorporated into the regenerated flagella. Agglutinin can be seen as a faint band near the top of the autoradiograph (*arrow*). The large *arrow* denotes the 350,000 M_r major membrane protein of the flagella.

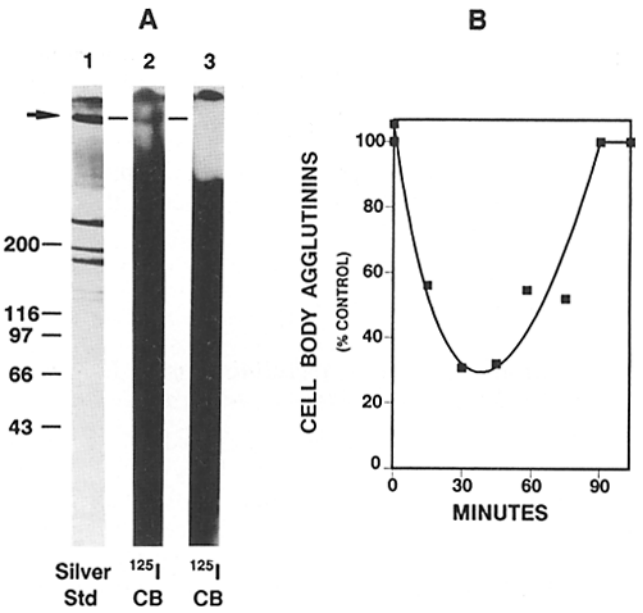

Figure 10. Depletion of radiolabeled cell body agglutinins during prolonged aggregation. (*A*) *Imp*-1 mt^+ cells were iodinated and allowed to regenerate their flagella under the conditions described in Fig. 9 *A*. An equal number of unlabeled mt^- gametes was mixed with these cells and allowed to agglutinate 45–60 min. Cells were deflagellated and the cell bodies were analyzed by 2.2–20% SDS-PAGE followed by autoradiography (lane *3*). The agglutinin band is absent in this autoradiograph (*arrow*). Lane *2* is the autoradiograph of agglutinin (*arrow*) of cell bodies from re-flagellated, radiolabeled cells incubated with an equal number of unlabeled *imp*-1 mt^+ cells for a nonagglutinating control and lane 1 is a silver stain of known agglutinin (*arrow*). (*B*) *Imp*-1 mt^+ (1.5×10^8 gametes/ml) were mixed with an equal number of mt^- gametes and allowed to aggregate. At the times indicated samples were deflagellated and the cell bodies analyzed for levels of mt^+ agglutinin by the dried-spot bioassay. Results are expressed as percent of cell body agglutinin in control, unmixed mt^+ gametes, which was 5,120 units.

tion, shows that the agglutinin was labeled under these conditions. Lane *2* is a silver stain of a sample of partially purified cell body agglutinin (*arrow*) for comparison. In control mixing experiments (not shown) we found that the iodinated agglutinin and partially purified, unlabeled agglutinin co-migrated in SDS-PAGE.

Chlamydomonas cells regenerate new flagella after deflagellation (Randall et al., 1967; Rosenbaum et al., 1969), and the newly regenerated flagella are fully agglutinable (Solter and Gibor, 1978). To test whether the cell body agglutinins were a source of flagellar agglutinins during regeneration, flagella that regenerated from iodinated cell bodies were isolated and analyzed by SDS-PAGE and autoradiography. The results shown in Fig. 9 *A*, lane *3*, indicated that the newly regenerated flagella contained a radiolabeled molecule that comigrated with agglutinin (*arrow*) indicating that flagella incorporated pre-existing, surface-labeled cell body agglutinins onto their newly forming membrane.

Other proteins that comigrated with flagellar proteins were also labeled on the cell body and moved onto the flagella during regeneration, most notable being the 350,000 M_r major membrane protein of the flagellum. Lane *2* of Fig. 9 *B* shows a shorter exposure of the sample in Fig. 9 *A*, lane *3*. The small upper *arrow* on the right in Fig. 9 *B* indicates the agglutinin band and the large *arrow* indicates the most highly labeled protein in the flagella, which co-migrated with the 350,000 M_r major membrane protein (Fig. 10 *B*, lane *1*).

Having shown that preexisting cell body agglutinins moved onto the flagella during flagellar regeneration, we also wanted to determine if the iodinated cell body agglutinins would be depleted during prolonged aggregation. Previously we and others have shown that the agglutinin pool is depleted during prolonged aggregation between impotent mt^+ gametes, which can agglutinate but not fuse, and wild-type mt^- gametes (Snell and Moore, 1980; Saito et al., 1985). Fig. 10 *B* shows an experiment with unlabeled gametes in which the levels of mt^+ cell body agglutinins were measured at various times after mixing *imp*-1 mt^+ gametes with mt^- gametes. The preexisting cell body agglutinins were significantly depleted (~75% loss) 35 min after mixing, at which time protein synthesis began to replenish the lost agglutinins, which returned to their premixing levels by 90 min. In the absence of protein synthesis the cell body agglutinin levels fall to zero (data not shown; Saito et al., 1985). Fig. 10 *A*, lane *2* is an autoradiograph of a control sample in which cell bodies from iodinated *imp*-1 mt^+ gametes were mixed with

unlabeled *imp*-1 mt^+ gametes, and lane *3* shows the cell bodies from iodinated *imp*-1 mt^+ gametes mixed with unlabeled mt^- gametes. (Fig. 10 *A*, lane *1* is a silver-stained sample of partially purified agglutinin [*arrow*] run on the same gel.) The cell bodies of the *imp*-1 mt^+ gametes that had been mixed with mt^- gametes showed complete loss of the radiolabeled agglutinin molecules, whereas the control cells retained the agglutinin.

Discussion

Localization of Cell Body Agglutinins on the External Surface of the Cell Body Plasma Membrane

The experiments reported here were carried out to characterize *Chlamydomonas reinhardtii* cell body agglutinins and to learn more about their movement to the flagellar surface. We found that cell body agglutinins and flagellar agglutinins are indistinguishable by several criteria. They have identical M_r by SDS-PAGE, exhibit the same elution properties on a hydrophobic interaction column, and the sedimentation coefficient of 12.3S for cell body agglutinins is similar to the 12S value reported for the flagellar agglutinins extracted by EDTA (Adair, 1985). We also have shown that both types of agglutinins are on the outer surface of their respective membranes, clarifying earlier conflicting studies (McLean and Brown, 1974; Snell, 1976*b*; Solter and Gibor, 1978; Saito et al., 1985; Goodenough, 1989).

This somewhat surprising result requires a new model to explain the lack of adhesiveness of cell bodies. Their nonadhesiveness can no longer be ascribed to the absence of cell surface agglutinins, but must derive from some other, as yet unknown, property of these molecules. One explanation for the functional inactivity of the cell body agglutinins is that their density is below some threshold concentration required for adhesion, as has been shown for other cell adhesion systems (Hughes et al., 1979; Weigel et al., 1979; Hoffman and Edelman, 1983). But, the cell body surface area (calculated by assuming that the cell body is a sphere with a diameter of 10 μm) is about nine times that of the flagellar surface (calculated by assuming that the two flagella are 11-μm-long cylinders with diameters of 0.5 μm). This ratio is the same as the ratio of cell body to flagellar agglutinins determined using the dried-spot bioassay. Assuming that there is a random distribution of agglutinins on the two domains, this means that the ratios between surface areas and agglutinins detectable in the in vitro dried-spot bioassay are similar on both domains. Of course, agglutinin-rich domains on the flagella but not the cell body could account for adhesiveness of only the flagella, and there is some evidence that agglutinins on the flagella are in linear arrays in *C. reinhardtii* (Goodenough, 1989) and *C. eugametos* (Tomson and Demets, 1989).

It is also possible that there is a posttranslational modification that confers activity on flagellar agglutinins. Consistent with this idea are the results from van den Ende's laboratory showing that flagellar agglutinins in *C. eugametos* can exist in an inactive form, depending on how the cells are illuminated (Kooijman et al., 1988) or in an apparent hyperactive form induced as the two gametes interact (Tomson et al., 1990). More recently we have shown that flagellar agglutinins in *C. reinhardtii* are inactive in situ immediately after zygote formation (Hunnicutt, 1989). If the cell body agglutinins indeed are in an inactive conformation, they may not have their typical rod-shaped structure, and would not be recognized by quick-freeze, deep-etch microscopy (Goodenough et al. 1985; Goodenough, 1989).

Even though they are nonfunctional in vivo, the discovery of cell body agglutinins was possible because they can be detected by use of the in vitro, dried-spot bioassay. We are not certain what features of the assay permit the agglutinins to be detected, and there may be agglutinin precursors within the cell body that are not active in the dried-spot bioassay. The cell body agglutinins might not function in vivo because they are not associated with the proper plasma membrane or cytoskeletal proteins. These putative, associated molecules might be found only on the flagella and could be required to render the agglutinins functional in vivo. If the cell body agglutinins are nonfunctional in vivo because of their conformation, then they may be activated during cell disruption or the denaturation that must occur concomitant with immobilization onto glass for the dried-spot bioassay. Further information about the molecular basis of activity of agglutinins in vivo and in the dried-spot assay can be investigated when new antiagglutinin mAbs become available.

Our results on the cell surface location of cell body agglutinins differ from studies on *Chlamydomonas eugametos* in which it has been shown that in addition to a cell surface pool of cell body agglutinins (Pijst et al., 1983) there is an internal pool representing as much as 20% of the total cell body agglutinins (Musgrave et al., 1986; Tomson and Demets, 1989). Since we find no evidence for an internal pool that can be detected with the dried-spot assay, this may be another example of the many differences between gametes of these two species.

Movement of Agglutinins from the Cell Body to the Flagella during Flagellar Regeneration

We show that the cell body agglutinins serve as a reservoir for flagellar agglutinins during regeneration. Moreover, the 350,000-M_r major membrane protein of the flagellum (Witman et al., 1972) is shown to reside on the cell body plasma membrane and to move onto the flagellum during flagellar regeneration. The finding that at least two flagellar membrane proteins are also on the plasma membrane of the cell body raises the possibility that most, if not all, flagellar membrane proteins are also on the cell body.

Presumably, as flagella regenerate, new membrane flows from the cell body to the flagella to accommodate the rapid increase in flagellar surface area. These results are consistent with experiments from Bouck's laboratory, showing that some high molecular flagellar proteins in *Euglena* also are found on the cell body plasma membrane (Rogalski and Bouck, 1982; Geetha-Habib and Bouck, 1982). Our results do not exclude the possibility that some proteins also are inserted into the flagellar membrane via membrane fusion (Bloodgood, 1982) or even that under some conditions agglutinins and the major membrane protein arrive on the flagella by membrane fusion. In this regard, Bouck (1971) showed that *Ochromonas* mastigonemes can be detected by thin-section EM not only on the flagellar surface but also in the Golgi apparatus and in membrane vesicles near the bases of the flagellum during flagellar regeneration. These organelles were not detected at other locations on the cell surface.

Our results suggest that there must be a mechanism for moving agglutinins from the cell body membrane to the flagellar membrane. Since the pool is external and the two membrane domains are physically contiguous, it is more likely that the movement occurs within the membrane. Possibly the same mechanism responsible for gliding motility and flagellar surface motility, visualized by the bidirectional movement of latex microspheres on the surface of the flagellum (Bloodgood, 1977), also moves molecules between these two domains.

A Functional Barrier to Movement of Agglutinins in Nonmating Gametes

In contrast to the apparently unrestricted movement of agglutinins from the cell body to the flagella during flagellar regeneration, we found that nonmating gametes have a functional barrier that makes cell body agglutinins unavailable for use on the flagella. When mt^+ gametes were incubated with mAb 2b40 they were rendered nonadhesive and no flagellar agglutinin activity was detected in the in vitro dried-spot bioassay. This loss of agglutinins is similar to the loss that occurs when mt^+ gametes bind to mAb 2b40-derivatized Sepharose beads. Cells bind to the beads, but then rapidly de-adhere, leaving their agglutinins on the antibody-derivatized beads (Snell et al., 1986). Together, these results indicate that interaction of the antibody with agglutinin leads to rapid release of the agglutinins from the flagella. Once the gametes were washed out of the antibody they rapidly regained flagellar agglutinins. But if these treatments were carried out in the presence of cycloheximide, an inhibitor of protein synthesis, the gametes did not recover their flagellar agglutinins. This requirement for protein synthesis indicates that there is a biosynthetic pathway for flagellar agglutinins. This pathway may reflect constitutive synthesis of agglutinins or associated proteins that normally replace lost molecules. Alternatively, the cells may detect the loss of these molecules and increase synthesis to replace them. In either case, the precise physical pathway followed by the agglutinins that appear on the flagella also is unknown. Membrane vesicles containing newly synthesized agglutinins may fuse directly with membrane at the bases of the flagella, or they may fuse first with the cell body plasma membrane and then the agglutinins may move onto the flagella.

Because we and others had previously shown the cellular pool of agglutinins (i.e., the cell body agglutinins) moved to the flagella as the flagellar agglutinins are lost during mating (Snell and Moore, 1980; Saito et al., 1985; Goodenough, 1989), we anticipated that this also would be true for nonmating gametes. Surprisingly, cells whose flagellar agglutinins had been depleted by incubation in CH and mAb 2b40 retained a full complement of cell body agglutinins. This indicated that in the absence of a signal the biosynthetic pathway is the only mechanism for restoration of flagellar agglutinins. That is, in nonmating gametes preexisting cell body agglutinins are unavailable for use on the flagella.

We do not yet know the molecular basis for this functional barrier, but Bloodgood (1988) has presented preliminary evidence for the existence of a barrier to movement of a surface antigen from *C. reinhardtii* cell bodies to flagella in vegetative cells. Musgrave et al. (1986) in their studies on *C. eugametos* also presented evidence for a functional barrier between the cell body and flagellum of gametes. The barrier in *C. eugametos* was of a different nature, however, in that it was not regulated during the mating reaction. The preexisting cell membrane agglutinins did not move from the cell body to the flagella of this species during flagellar regeneration or sexual signaling (Musgrave et al., 1986). On the other hand, this group more recently reported evidence consistent with an internal pool of agglutinins in *C. eugametos* that can move to the flagella during signaling (Tomson and Demets, 1989).

Although the nature of the barrier in *C. reinhardtii* is unknown, it may be that the necklace (Gilula and Satir, 1972) or bracelet of intramembranous particles that surrounds the base of the flagellum provides a physical barrier as suggested by Weiss et al., (1977). An alternative possibility would be that the cell body agglutinins are associated in some way with the cytoskeleton, thereby restricting their movement (Friend, 1989; Gumbiner and Louvard, 1985).

A similar segregation of cell adhesion molecules into distinct membrane domains prior to gametic interactions has been demonstrated in sperm of higher organisms (Friend, 1989; Myles and Primakoff, 1984; Cowan et al., 1986, 1987; Primakoff et al., 1985, 1987; Phelps et al., 1988; Lopez and Shur, 1987) and may be a common mechanism for sequestering these critical molecules until the gametes are activated in preparation for fusion (Kopf et al., 1986; Endo et al., 1988; Saling and Storey, 1979; Florman and Storey, 1982; Wasserman, 1987).

In summary, we have shown that adhesion molecules in *Chlamydomonas* are segregated into two surface regions: a flagellar membrane domain and a cell body plasma membrane domain. Cell body agglutinins can move onto the flagella during flagellar regeneration and during sexual signaling, but a functional barrier prevents movement in the absence of a signal. Future experiments will be directed to elucidating the molecular basis of this functional barrier in *Chlamydomonas reinhardtii* and the mechanism of activation of agglutinins as they move from the cell body to the flagella.

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