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 *mr* 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 *mr* 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*, 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 (*mr* and *mr*) 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 *mr* mutant (*imp*-l) 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.
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-dimethoxyisouquinoline), 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). 

Cycloheximide (CC-1690), 6145c (mt+) [CC-1691] and the nonmutating, 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, 1976a) by transferring vegetative cells (4.7 × 10⁹ 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 KH₂PO₄ and 0.3 g/liter of K₂HPO₄.

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-Calculator buffer (HC; 10 mM Hepes, 1 mM CaCl₂, 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 3.0 rpm (1800 gₑₘ) 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 gₑₘ) 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 × 10⁶ 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 cell; 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 10 mM, respectively. These samples were then centrifuged at 43,000 rpm (145,000 gₑₘ) 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 gₑₘ) 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 bodies 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/10⁷ cells with 10% of the agglutinin activity associated with the flagella and 90% of the agglutinin activity associated with the cell bodies.

The Journal of Cell Biology, Volume 111, 1990 1606
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) 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), 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 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 SPW 7.5 × 75 mm column with guard column (Beckman Instruments), equilibrated with 1.0 M AS in 0.1 M K2HPO4, 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 K2HPO4, 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 gs) for 3.75 hr 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 × 107 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 OD435 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 × 109 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 121I were mixed with the cells and the solution placed in an ice-water bath. Lactoperoxidase (1 mg/ml in HC) and hydrogen peroxide (diluted 1:1000 in HC) were added to the cell-iodide solution by the following protocol: 30 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 gs) for 5 min at 25°C in a tabletop centrifuge (rotor 958; Darnan/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. 10B). 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 (Hoefler Scientific Instruments, San Francisco, CA), placed on preflashed Kodak XAR film in a cassette with two intensifying screens (Cronex Lightning-plus, 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, IBI, 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, 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-
Molecular sieve chromatography of cell body agglutinin on BioGel A-15M. Partially purified cell body agglutinin extract from \(5 \times 10^{11} m^+\) 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 Mr polypeptide that copurified with agglutinin activity. The numbers along the side of the gel represent the Mr of the molecular weight standards.

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 agglutinin activity (top) again copurified with the high Mr 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 Mr 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 Mr 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 \(m^+\) and \(m^-\) 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.
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 dibutylryl 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.

**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 flagellar 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 agglutinin 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-
induced depletion of agglutinins required protein synthesis, we repeated the experiment in the presence of the protein synthesis inhibitor cycloheximide. Gametes (3 +) 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.

**Figure 4.** The effects of CH on recovery of adhesiveness after treatment of (3 +) gametes with mAb 2b40. (3 +) gametes (107 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 (3 +) 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 (3 +) gametes with mAb 2b40 and CH. (3 +) cells (107 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 (cross-hatched 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.

**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, (3 +) gametes were incubated with mAb 2b40 and CH for 180 min at which time the Coulter counter assay showed that the (3 +) 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 (3 +) 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 (3 +) gametes were depleted of their flagellar agglutinins by incubation in mAb 2b40 and CH for 180 min.
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 sexual signaling remained 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 allowed to regenerate flagella, and the cell bodies and flagella then were analyzed by SDS-PAGE and autoradiography. Fig. 9 A (lane I), the autoradiograph of the cell body fractions, showed that cells that had been incubated with dibutyryl cAMP and papaverine or control buffers, were deflagellated and then were analyzed by SDS-PAGE and autoradiography. The autoradiograph of the cell body fractions showed that cells that had been incubated with dibutyryl cAMP and papaverine had high levels of cell body agglutinin levels of gametes treated with mAb 2b40 and CH. Mt+ gametes pretreated 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 x 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 appropriate samples 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%.

**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 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 I), the autoradiograph of the cell body frac-
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 samples, 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 Mr, major membrane protein of the flagella.

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 Mr, 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 mr ga-

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.

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, radio-labeled 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 x 10⁵ gametes/ml) were mixed with an equal number of unlabeled 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.
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 Mr, 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, 1976b; 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 non-adhesiveness 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 agglutinins (Musgrave et al., 1986; Tomson and Demets, 1989).

TheJournal of Cell Biology, Volume 111, 1990

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-Mr 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 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 weight protein in Euglena can be detected, and there may be agglutinin precursors within the cell body that are not active in the 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 anti-agglutinin 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.

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., 1983; 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 anti-agglutinin mAbs become available.
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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