

Wheat Germ Agglutinin Induces Mating Reactions in *Chlamydomonas eugametos* by Cross-Linking Agglutinin-associated Glycoproteins in the Flagellar Membrane

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Abstract. Species-specific binding between the flagellar surfaces of mating types plus and minus (mt^+ and mt^-) gametes of *Chlamydomonas eugametos* is mediated by mating type-specific agglutinins. Their interaction triggers several mating responses that are necessary for cell fusion, such as flagellar twitching, flagellar tip activation, redistribution of agglutinin molecules to the flagellar tip (tipping), and mating structure activation. Earlier, we reported that a monoclonal antibody (mAb 66.3) can induce mating reactions by cross-linking the agglutinins (Homan, W. L., A. Musgrave, H. de Nobel, R. Wagter, A. H. J. Kolk, D. de Wit, and H. van den Ende. 1988. *J. Cell Biol.* 107:177-189). Here we report that the lectin wheat germ agglutinin (WGA), which does not bind to the agglutinins, can also invoke all these mating reactions. We show, by immunofluorescence studies using anti-WGA and an agglutinin-specific monoclonal anti-

body (mAb 66.3), that WGA induces the redistribution of agglutinin to the flagellar tips of mt^- gametes. Vice versa, when agglutinin tipping is induced by mAb 66.3, the WGA-binding glycoproteins are also tipped. Under the same conditions, the major flagellar glycoproteins are not redistributed, indicating that membrane transport is limited to a few components. We conclude that each agglutinin is associated with a WGA-binding glycoprotein. When cells lacking agglutinin or cells possessing inactive agglutinins are treated with WGA, mating responses are again elicited.

The data suggest that clustering of agglutinin-containing complexes results in the production of intracellular signals, such as cAMP, and the coupling of the complex to a force generating system. In nature, the complexes are clustered via the agglutinins, but artificially they can be clustered by lectins or antibodies directed against other proteins in the complex.

THE unicellular green alga *Chlamydomonas eugametos* provides a good model system for studying cellular recognition and intercellular communication. Sexual reproduction is initiated by a sex- and species-specific adhesion between the flagellar membranes of mating types plus and minus (mt^+ and mt^- , respectively)¹ gametes that results in the aggregation of cells (sexual agglutination). For each mating type, a high molecular weight glycoprotein (agglutinin) is responsible for this adhesion. They are long, linear molecules extrinsically bound to the flagellar membrane (Musgrave et al., 1981; Homan et al., 1982; Klis et al., 1985; Crabbendam et al., 1987; for similar information about the agglutinins of *C. reinhardtii* see Adair et al., 1982; Collin-Osdoby and Adair, 1985). Agglutinin interaction has a signaling function since it elicits the formation of a plasma

papilla (mating structure) in each of the agglutinating cells by which the gamete eventually fuses with a partner. In *C. eugametos*, this naked papilla penetrates the cell wall between the bases of the flagella. In *C. reinhardtii*, however, the cell wall is degraded by a periplasmic autolysin (Claes, 1971; Snell, 1982; Matsuda et al., 1987) and the gamete protoplasts are released before fusion takes place. Sexual agglutination also initiates some flagellar events that are important for cell fusion. Normal swimming is arrested (Homan et al., 1980) and agglutinins are redistributed to the flagellar tip (Goodenough et al., 1980; Homan et al., 1987, 1988). This redistribution plays an important role in flagellar realignment and the sorting of agglutinating gametes into pairs with mutually aligned papillae (for example see Musgrave and van den Ende, 1987). A third response, called flagellar tip activation (FTA), involves a change in the flagellar tip morphology (Mesland et al., 1980; Elzenga et al., 1982; Crabbendam et al., 1984), but the function of this phenomenon is unknown.

We assume that the mt^+ and mt^- agglutinins bind to each

1. *Abbreviations used in this paper:* GTC, guanidine thiocyanate; MSA, mating structure activation; mt^- , mating type minus; mt^+ , mating type plus; FTA, flagellar tip activation; WGA, wheat germ agglutinin.

other and not to other flagellar receptors. This view is based on the fact that a monoclonal antibody, designated mAb 63.3, binds to the sexual site of the mt^- agglutinin and prevents agglutination (Homan et al., 1988). In such a unipolar system, both agglutinins are not only involved in adhesion but also in signal transduction and generation. This is illustrated by the fact that isolated mt^- agglutinin induces FTA and twitching in mt^+ gametes (Homan et al., 1980; Elzenga et al., 1982). Yet when the same agglutinin molecules on mt^- gamete flagella are cross-linked by mAb 63.3, all the mating reactions are induced (Homan et al., 1988). This supports the original suggestion of Goodenough and Jurivich (1978) that, in *Chlamydomonas* gametes, agglutinin clustering is an important step in the generation of a signal that elicits secondary sexual responses.

Lectins are also polyvalent proteins capable of binding to and cross-linking cell surface glycoproteins. The ability of concanavalin A to bind *Chlamydomonas* flagella is well documented (Wiese and Shoemaker, 1970; Mclean and Brown, 1974; Claes, 1977; Musgrave et al., 1979b; Mesland et al., 1980). It has been shown to induce FTA (Mesland et al., 1980), cell wall release (Claes, 1977), and the tipping of glycoproteins (Bloodgood et al., 1986) in *C. reinhardtii*, all of which are characteristic of a sexual response. In *C. eugametos*, it binds the mt^+ sexual binding site without inducing such a response (Musgrave et al., 1979b). However, we now report that all the mating reactions can be induced in both mating types of *C. eugametos* by wheat germ agglutinin (WGA) without binding the agglutinins but by binding to two other glycoproteins. When mt^- gametes are treated with WGA, not only the WGA-binding glycoproteins, but also the agglutinins, are redistributed to the flagellar tip, which indicates that the mt^- agglutinin and WGA-binding glycoprotein are complexed together in the flagellar membrane. This suggests that WGA can circumvent the need for agglutinins in inducing sexual responses. In support, we show that cells lacking active agglutinins respond sexually to WGA.

Materials and Methods

Cell Cultures

The *C. eugametos* strains UTEX 9 (mt^+) and UTEX 10 (mt^-) were obtained from the Culture Collection of Algae (University of Texas at Austin, Austin, TX). The strains 17.17.2 (mt^+) and 5.39.4 (mt^-) were obtained by crossing UTEX 9 with UTEX 10 (Shuring et al., 1987). The cells were grown in Petri dishes on an agar-containing medium as described earlier (Mesland, 1976). Gamete suspensions were obtained by flooding 2–4-wk-old cultures with 10 mM Hepes buffer, pH 7.6.

When cells were grown in liquid cultures, an N-free medium was used (Kates and Jones, 1964; Tomson et al., 1985). The cells were cultivated in 300 ml medium at 19°C in glass tubes (3.5 cm diameter) and aerated with compressed air containing 5% CO₂. They were illuminated with white fluorescent light (150 μ E s⁻¹m⁻²). A regime of 16 h in the light and 8 h in the dark was used.

Materials

The WGA used was purchased from Serva (Heidelberg, FRG), but we found that WGA from other sources (Calbiochem-Behring Corp., La Jolla, CA; Sigma Chemical Co., St. Louis, MO) bound the same flagellar glycoproteins and induced the same reactions in live cells. TRITC-labeled rabbit anti-WGA and FITC-conjugated WGA were prepared as described by Mishel and Shiigi (1980). Rabbit anti-WGA was obtained from Sigma Chemical Co.

Flagella, Flagellar Membranes, Agglutinins, and Cell Walls

Flagella were amputated from the cells by pH shock (Witman et al., 1972) and separated from the cell bodies as described previously (Kooijman et al., 1986). Flagellar membrane vesicles were isolated as described by Homan et al. (1980). Cell walls were isolated by vortexing cell bodies with glass beads (0.5 mm diameter) and subsequently purified as described by Musgrave et al. (1979a). mt^- agglutinin was obtained from whole cells of UTEX 10 by guanidine thiocyanate (GTC) extraction and purified by gel filtration as described by Musgrave et al. (1981). mt^+ agglutinin was obtained from whole cells of strain 17.17.2 by extraction in 0.1% Triton X-100 and purified as described by Klis et al. (1985).

Extraction of Membrane Components

For Triton X-100 extraction, membranes were incubated in 1% Triton X-100 at 20°C for 30 min. The nonextractable part was sedimented by centrifugation at 50,000 g for 1 h. Thereafter, the detergent was removed from the supernatant by chromatography over a phenyl-Sephrose column (Pharmacia Fine Chemicals, Piscataway, NJ).

For extraction in GTC, membranes were treated with 3 M GTC for 30 min at 20°C. After centrifugation at 50,000 g for 1 h, the supernatant was dialyzed against PBS to remove the GTC.

Quantification of Agglutinin Activity

The biological activity of extracted agglutinin was determined using the charcoal test (Musgrave et al., 1981). An extract was serially diluted and each dilution was treated with activated charcoal to adsorb the agglutinin. Subsequently, the charcoal was washed three times in water and tested with gametes of the opposite mating type to see if they agglutinated to the charcoal. The highest dilution that was active is the titer of the extract. The total amount of agglutinin in an extract is expressed in units (titer \times volume of the extract in milliliters).

Mating Reactions

FTA was observed and quantitated as described previously (Kooijman et al., 1986).

Twitching was assessed microscopically within 1 min after the addition of the WGA solution to gametes. Tipping of WGA-binding glycoproteins was detected using an indirect immunofluorescence test (Kooijman et al., 1986) or FITC-conjugated WGA.

Mating structure activation (MSA) was observed by indirect immunofluorescence assay using WGA at a concentration of 500 μ g/ml. WGA binds to the exposed papillar membrane and was observed as a fluorescent point between the flagellar bases.

Cells were tested for local cell lysis by enclosing a suspension of gametes on a microscope slide under a coverslip. After 5–10 min, the preparation had dried out to the extent that the cell contents were squeezed out, resulting in the extrusion of a "balloon" between the bases of the flagella (Schuring, F., personal communication).

cAMP Determination

For cAMP measurements, 100- μ l aliquots (containing 1–2 \times 10⁶ cells) were extracted in 3 M PCA as described by Pijst et al. (1984). The amount of cAMP was determined using a competition radioimmunoassay based on the method of Steiner et al. (1972). For this assay, a cAMP-specific antiserum and [¹²⁵I]-cAMP tyrosine methyl ester as a competing agent (Amersham International, Amersham, UK) were used. Acetylation of cAMP, using acetic anhydride and triethylamine, resulted in a sensitivity of 2 fmol cAMP per tube. By incubating the samples with phosphodiesterase, it was shown that the measured values represented cAMP.

Double-labeling Immunofluorescence Tipping

Tipping, the redistribution of flagellar membrane components to the flagellar tips, was induced by 50 μ g/ml WGA. After a 30-min incubation, the cells were fixed in 1.25% glutaraldehyde for 30 min. After three washes in PBS, the agglutinin and bound WGA were labeled by incubation in PBS containing a 1:10 dilution of mAb 66.3 and a 1:100 dilution of TRITC-conjugated rabbit anti-WGA. After another 30-min incubation, the cells were washed

in PBS, and subsequently mAb 66.3 was visualized by a 30-min incubation in a 1:100 dilution of FITC-conjugated goat anti-mouse IgG (gamma and light chain specific; Tago Inc., Burlingame, CA). When tipping was induced by a 30-min incubation with mAb 66.3, a similar procedure was followed. The cells were examined under a microscope (Carl Zeiss, Oberkochen, FRG) fitted with a fluorescence attachment. FITC and TRITC were selectively visualized using the filter (Carl Zeiss) combinations 14 and 09, respectively. Photographs were taken using RE 2475 film (Eastman Kodak Co., Rochester, NY).

Gel Electrophoresis and Western Blot Analysis

Samples of extracted material were lyophilized and dissolved in sample buffer, and the components were separated by SDS-PAGE according to Laemmli (1970) in 0.5-mm-thick, 8 × 8-cm slab gels containing a 2.2–20% acrylamide gradient. The gels were stained for glycoconjugates with periodic acid Schiff reagent (Zacharias et al., 1969). Electroblot transfer of protein from the gel onto nitrocellulose (Sartorius, Breukelen, The Netherlands) was performed as described by Towbin et al. (1979). Subsequently, the nitrocellulose was blocked in 3% BSA in PBS for 1 h at 40°C. The blots were incubated in the appropriate dilution of antibody in PBS with 3% BSA. After a 50-min incubation at 20°C, the blots were washed five times for 10 min in washing buffer (20 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1 mg/ml gelatine, 0.15 M NaCl, 0.05% [wt/vol] Tween 20). The blots were incubated in a 1:1,000 dilution of peroxidase-conjugated goat anti-mouse IgG (heavy and light chain; Tago Inc.) at 20°C for 50 min. After five washing steps as described above, the blots were incubated in 50 mM acetate buffer, pH 4.5,

containing 0.5 mM 3-amino 9-ethylcarbazole and 0.03% H₂O₂. After color development, the blots were washed in 50% ethanol and immediately photographed. For the identification of glycoproteins binding WGA, a similar procedure was used. Blots were incubated with 10 µg/ml WGA. The WGA was visualized using a 1:100 dilution of rabbit anti-WGA and a 1:1,000 dilution of peroxidase-conjugated goat anti-rabbit IgG (Miles Yeda, Rehovot, Israel).

Results

Induction of Mating Reactions

In a normal mating reaction between mt⁻ and mt⁺ gametes, a number of cellular responses are elicited by the interaction of the flagella: twitching, FTA, tipping, and the formation of a plasma papilla. These reactions are also evoked when a single cell type is presented with agglutinin-directed mAb 66.3, and, in this report, we shall illustrate a similar reaction for cells treated with lectin WGA.

Tipping. The binding of WGA to gametes could be observed using an indirect immunofluorescence assay as illustrated in Fig. 1 A. The fixed flagella were uniformly labeled. WGA did not bind to the flagella in the presence of 20 mM

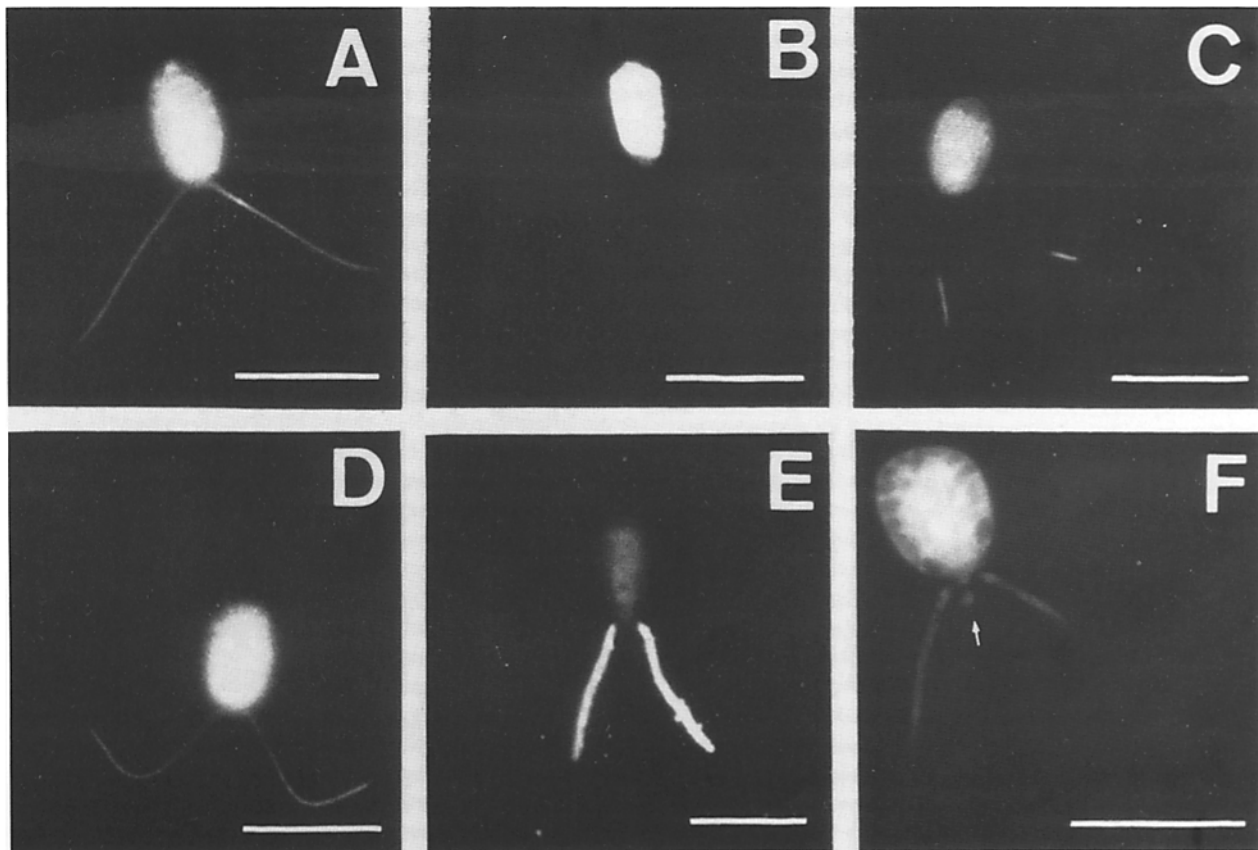


Figure 1. Immunofluorescence pictures of WGA-treated gametes. Fixed cells of strain 5.39.4 were treated with 50 µg/ml WGA at 20°C (A), and bound lectin was visualized using rabbit anti-WGA and FITC anti-rabbit. Live cells were treated with 50 µg/ml WGA at 20 (C) or 5°C (D) for 30 min before fixing them and again visualizing the bound WGA by indirect immunofluorescence. In C, the WGA-binding compounds were tipped. When 25 mM *N*-acetylglucosamine was present during the WGA-incubation, the lectin was not bound (B). Live gametes, treated with a high concentration of WGA (500 µg/ml), were induced to form papillae which were visualized by the indirect immunofluorescence technique (F). Live gametes treated with mAb 44.2, which binds several flagellar glycoproteins but not the agglutinin, were seen to be uniformly labeled when fixed and treated with FITC anti-mouse (E). In all cases, the fluorescence of the cell body is due to the autofluorescence of the chloroplast. Bars, 10 µm.

Table 1. Induction of Mating Reactions by WGA and Antibodies

	Sexual agglutination		WGA		mAb 66.3		Fab 66.3	mAb 44.2
	mt ⁻	mt ⁺	mt ⁻	mt ⁺	mt ⁻	mt ⁺	mt ⁻	mt ⁻
Twitching	+	+	+	+	+	+	-	-
FTA	+	+	+	+	-	-	-	-
Tipping	+	+	+	+	+	+	-	-
MSA	+	+	+	+	+	+	-	-

The effects of WGA and mAb 66.3, described in Fig. 2 and Homan et al. (1988), respectively, are summarized in this table. Furthermore, Fab fragments of mAb 66.3, obtained as described by Homan et al. (1988), were tested for induction of mating reactions at the same protein concentration that was used for the bivalent antibodies. The effect of a monoclonal antibody directed against the major flagellar glycoproteins, mAb 44.2, was also tested using a 2-, 10-, 50-, 250-, and 1,500-fold dilution of a standard antibody solution. Binding of this antibody in a twofold dilution to the flagella is shown in Fig. 1 F.

N-acetylglucosamine, which indicates that the lectin bound specifically to *N*-acetylglucosamine residues on flagellar glycoproteins (Fig. 1 B; Goldstein et al., 1975). When 50 μ g/ml WGA was added to live gametes at 20°C, the lectin bound to the flagella and was redistributed to the flagellar tips (Fig. 1 C), as we reported earlier for mt⁺ cells (Kooijman et al., 1986). Tipping was temperature dependent and not a consequence of the flagellar beating since no tipping took place at 5°C (Fig. 1 D) even though swimming was normal. Apart from agglutinins and WGA-binding glycoproteins, the flagellar membrane contains an abundant class of glycoproteins that are labeled by mAb 44.2 (Homan et al., 1987). These glycoproteins were not redistributed when cells were incubated with mAb 44.2, irrespective of the concentration used (Fig. 1 E and Table I). The transported WGA-binding glycoproteins were not accumulated at the extreme flagellar tips as described for agglutinin tipping (Homan et al., 1987). Instead, they accumulated in the distal 10–50% of the flagellar length. The size of the fluorescent WGA tip increased with WGA concentration. At concentrations >50 μ g/ml, the tip sometimes involved the whole length of the flagellum. As a consequence, tipping could only be quantitated up to a concentration of 50 μ g/ml WGA (Fig. 2, \square). After 50 min of WGA treatment, 90% of the cells exhibited tipping. This level was maintained for at least another 50 min (Fig. 3 A, \square). However, the amount of tipped material steadily diminished, resulting in a shorter fluorescent zone (Fig. 3 B) and suggesting that WGA-binding glycoproteins were shed into the medium. We do not have evidence for the transport of tipped material to the flagellar bases and its subsequent loss, as described by Bloodgood et al. (1986), for some paralyzed mutants of *C. reinhardtii*. When gametes were treated with 25 mM *N*-acetylglucosamine 90 min after the addition of WGA and fixed at subsequent intervals, all WGA was lost from the flagella within 2 min (data not presented). When new WGA was subsequently added, no binding at the tips occurred, suggesting that the WGA-binding glycoproteins had been relocated away from the tips. The kinetics of WGA tipping was comparable with agglutinin-induced tipping during sexual agglutination.

Flagellar Tip Activation. During sexual agglutination, FTA is complete within 10 min of mixing the gametes. This

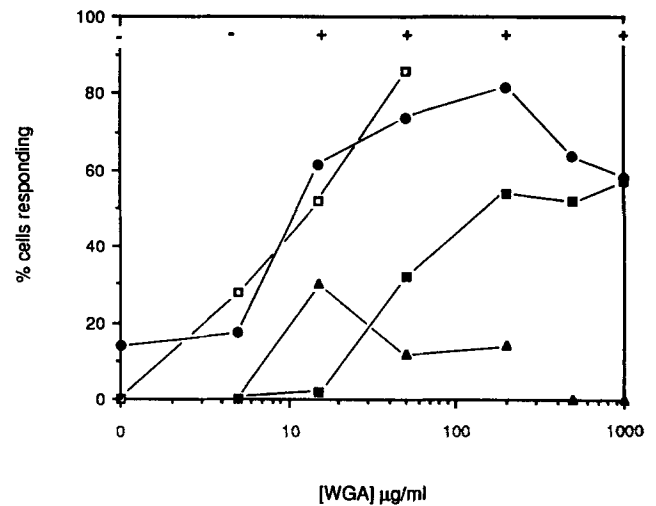


Figure 2. Effect of WGA concentration on the induction of mating reactions. Gametes of strain 5.39.4 were incubated with different WGA concentrations. After 15–60 s, the presence of twitching activity was determined by microscopical observation and is shown in the figure (+ and -). After 30 min, the cells were fixed in glutaraldehyde, and bound WGA was visualized using the indirect immunofluorescence test. The number of cells with tipped flagella was recorded (\square). A similar sample was tested for partial and complete FTA (\bullet and \blacktriangle , respectively), as described by Kooijman et al. (1988). The degree of MSA was determined by counting the number of cells that possessed mating structures after visualizing the papillae by immunofluorescence (\blacksquare).

reaction involves the elongation of the outer microtubules, which results in a rounding of the flagellar tip, and the accumulation of fibrous material (Mesland et al., 1980; Elzenga et al., 1982). FTA was induced in gametes of both mating types by WGA, but the process was less complete than during sexual agglutination. As depicted in Fig. 2, the majority of the flagella was only partially activated. The initial effect of WGA on flagellar tip morphology was rapid, within 2 min, but the appearance of fully activated tips was slow and only reached a maximum after 100 min (Fig. 3 A). The percentage of completely activated flagellar tips was maximal when a concentration of 20 μ g/ml WGA was used (Fig. 2, \blacktriangle). Like tipping, FTA was reversed when WGA was removed from the cells. This is remarkable because after sexual fusion the mt⁻ flagella remain activated even though the flagella de-adhere (Crabbendam et al., 1984). It seems likely that cell fusion has an effect on mt⁻ gametes that causes the FTA to persist.

Sexual Twitching and Isoagglutination. When the flagella of mt⁺ and mt⁻ gametes bind together, their normal swimming behavior stops and they start to twitch. In mt⁺ gametes, this reaction can also be evoked by the addition of mt⁻ agglutinin (Homan et al., 1980). The cells move to the surface of the suspension and lie there twitching without clumping together. In contrast, in mt⁻ gametes a twitching reaction is only induced by mt⁺ agglutinin when it is bound to particulate material, such as charcoal (Musgrave et al., 1981) or membrane vesicles. WGA in concentrations of 50–1,000 μ g/ml induced twitching in both mating types (Fig. 2, +). Within 5–10 s, some of the cells moved to the surface of the liquid where they lay twitching without agglutinating. Other cells became isoagglutinated by the WGA and twitched

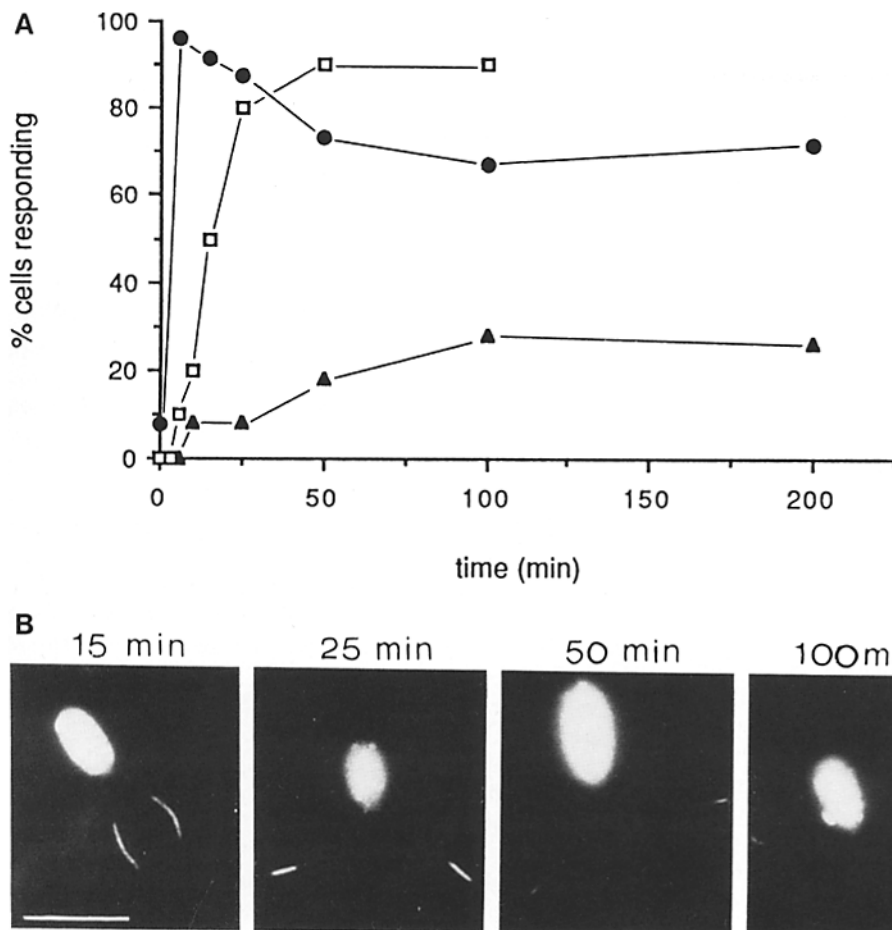


Figure 3. Kinetics of WGA-induced tipping and FTA. Gametes of strain 5.39.4 were incubated with 50 $\mu\text{g/ml}$ WGA. At the indicated times, samples were fixed in glutaraldehyde, and tipping and FTA were quantitated as described in Fig. 2. (A) Induction of tipping (□) and partial and complete FTA (● and ▲, respectively). (B) Immunofluorescence pictures showing the distribution of WGA-binding components after different incubation periods. Bar, 10 μm .

in a manner similar to gametes agglutinating with the opposite mating type. The fraction of isoagglutinating cells increased with the concentration of WGA, and, at a concentration $>500 \mu\text{g/ml}$, all the gametes, irrespective of mating type, were cross-linked.

MSA. In both mt^+ and mt^- gametes, the mating structure was activated by treatment with WGA. This was assessed in two ways. First, gametes were fixed during treatment, and the presence of mating structures (papillae) was detected by indirect immunofluorescence using WGA and anti-WGA. This is possible because the intact cell wall does not bind WGA, while the plasma membrane does; thus, when the plasma membrane protrudes through the cell wall, the presence of mating structures can be visualized (Fig. 1 F). While this test was effective, 60% of the gametes sometimes being registered as positive (Fig. 2, ■), the quantitative result varied strongly from one experiment to another. In the second method, the WGA-treated gametes were not fixed but were squeezed by letting them dry out between a microscope slide and a coverslip. The protoplast in the region of the papilla was then seen under phase-contrast optics to balloon outwards. Strictly speaking, this only demonstrates the presence of a hole in the cell wall, probably due to cell wall lytic activity, but this is an important part of the MSA response and is never found in untreated gametes. The number of cells that responded to WGA in this way was seldom $>30\%$, but the variation between experiments was appreciably less than with the WGA-fluorescence assay.

Agglutinins Are Not Necessary for WGA-induced Responses

Although fully competent gametes were used for studying the above responses, similar effects could be obtained with nonagglutinable "gametes." Two examples will be described: light-sensitive cells in the dark and cells flooded with NH_4Cl instead of water.

The *C. eugametos* strain 5.39.4 is light sensitive in that it is sexually incompetent in the dark. The flagellar agglutinins are present but inactive and thus cannot bind partner flagella. Each molecule is modified, resulting in a conformational change that masks the sexual binding site (Kooijman et al., 1988). In other respects, the cells are competent and, if treated with dibutyryl cAMP, are able to produce papillae (to be reported elsewhere). When nonagglutinable dark cells were treated with WGA, they responded sexually with the exception that no papillae were formed (Table II). The light-insensitive strain Utex 10 responded fully to WGA both in the light and dark.

When 2–3-wk-old agar cultures are flooded overnight with 0.5% NH_4Cl , the cells swim free as nonagglutinable gametes. While they resemble vegetative cells in being sexually incompetent, they do not divide within the first 24 h. The effect of NH_4Cl has not been established for *Chlamydomonas*, but it is likely that the pH of the endoplasmic reticulum is raised and that the synthesis of some glycoproteins is inhibited as has been shown in other systems (Winkler and

Table II. WGA-induced Mating Reactions in Agglutinable and Nonagglutinable Cells

	Control	NH ₄ Cl	Dark
Agglutination	+	-	-
Binding of mAb 66.3	+	-	-
mAb 66.3-induced twitching	+	-	-
WGA-induced twitching	+	+	+
WGA-induced FTA	+	+	+
WGA-induced tipping	+	±	+
WGA-induced cell wall lysis	+	-	-

Nonagglutinable cells were obtained by placing a light-sensitive strain in the dark for 1 h or by flooding agar plates with 0.5% NH₄Cl instead of Hepes buffer. These cells were tested for agglutination activity under the light microscope after adding gametes of the other mating type. The ability to bind mAb 66.3 was tested using the indirect immunofluorescence test as described in Kooijman et al. (1988). Cells were tested for twitching, FTA, and tipping, as described in Fig. 2, using 50 µg/ml WGA. For mAb 66.3-induced twitching, a 2 and 20× diluted solution of the antibody was used. The ability of cells to perform cell wall lysis was tested by incubation with 500 µg/ml WGA for 30 min. Cell wall lysis was observed by extrusion of the plasma membrane between the bases of the flagella after the cells were dried out between a coverslide and object glass. Nonagglutinable cells were also tested using a WGA concentration of 2 mg/ml.

Grainger, 1978; Wagner et al., 1986). What has been established for *C. eugametos* is that the flagella of such cells are devoid of agglutinins even though the major glycoproteins are present (Musgrave et al., 1981). Remarkably, their flagella were labeled by WGA just as effectively as gamete flagella and the cells responded sexually as noted in Table II. We conclude that agglutinins are not essential for the induction of a sexual response and that WGA induces its effects via another flagellar component.

WGA-induced cAMP Response

Since a role for cAMP has been implicated in signaling during mating in *Chlamydomonas* (Pijst et al., 1984; Pasquale and Goodenough, 1987), we investigated whether WGA could induce an increase in the cAMP level in gametes. As depicted in Fig. 4, the cAMP concentration in an agglutinating mixture of mt⁺ and mt⁻ cells increased about sevenfold

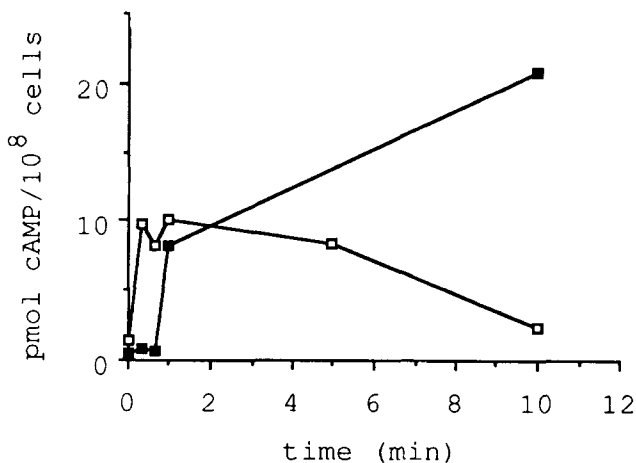


Figure 4. Agglutination- and WGA-induced changes in the cAMP levels in gametes incubated in the light. cAMP concentrations were determined after mixing mt⁺ and mt⁻ gametes (□) or during the incubation of mt⁻ gametes in 500 µg/ml WGA (■). The cAMP levels measured during agglutination are the mean levels in the mt⁻ and the mt⁺ cells.

within 20 s. This level was maintained over the next 4 min and was followed by a decrease. The cAMP levels are mean values for the mt⁻ and mt⁺ gametes, which have been shown to exhibit a similar time course in cAMP production (Pijst et al., 1984). The WGA-induced increase in cAMP concentration in the mt⁻ cells was as fast as in agglutinating cells, but occurred after a lag period of 40 s. After 1 min, the same level was reached, but the increase continued to a level twice as high as that in agglutinating cells.

WGA-binding Components in the Flagellar Membrane

The fact that sexual responses can be evoked by polyvalent antibodies is thought to be due to cross-linking flagellar surface components, the agglutinins in particular (Goodenough and Jurivich, 1978; Homan et al., 1988). The action of WGA may also be due to cross-linking, but the effect on NH₄Cl cells indicates that this does not take place via the agglutinins. On the other hand, mAb 44.2, which binds the major flagellar glycoproteins of mt⁻ cells (Fig. 6 A; Homan et al., 1987) but not the agglutinins, is totally without biological effect. Thus, an effect can only be produced by cross-linking certain glycoproteins. We therefore asked which ones are bound by WGA?

First, we tested whether or not the glycoproteins are gamete specific. In vegetative liquid cultures, all the cells are sexually incompetent and devoid of agglutinins until the nutrients are exhausted and then, at the end of the exponential phase of growth, some of the cells differentiate into gametes (Tomson et al., 1985). Using such cultures, the ability of the flagella to bind WGA was tested. In the experiment illustrated in Fig. 5, gametogenesis took place on day 4, when 30% of the cells were found to be mating competent. The next day, competence had returned to zero. The binding of WGA to these cells (Fig. 5 A) was correlated with gametogenesis whereas the ability to bind mAb 44.2 was not. All mt⁻ cells, irrespective of their state, bound mAb 44.2 (Fig. 5 B).

Using Triton X-100 extracts of gamete flagella, Western blot analysis was performed to identify WGA-binding proteins. Two were identified, characterized by a molecular masses of 19 and 123 kD. There was considerable variability in the intensity of these bands from different batches of cells (Fig. 6 A, lanes 3 and 4), however, they were generally detected in all cells. This variation is similar to that of the agglutinin since not all gametes are equally agglutinable. Using the more sensitive immunogold labeling method, some minor WGA-binding glycoproteins could also be detected. The major WGA-binding glycoproteins did not bind mAb 44.2 (Fig. 6 A, lane 5). Significantly, the mt⁻ agglutinin band, which clearly bound mAb 66.3 (Fig. 6 A, lane 2), was never found to bind WGA, even when concentrated purified agglutinin was used (Fig. 6 B, lane 3). Similarly, the mt⁺ agglutinin, which also binds mAb 66.3 (Homan et al., 1988), failed to bind WGA (Fig. 6 B, lane 6).

The 19- and 123-kD glycoproteins could be extracted from flagellar membrane vesicles into 1% SDS. Although both could also be extracted by 1% Triton X-100, the 123-kD component was not completely extracted. The 19- but not the 123-kD glycoprotein could also be extracted by chaotropic reagents, such as 3 M GTC, that do not dissolve the lipid membrane. These data suggest that the 123-kD glycoprotein is an intrinsic membrane component, while the 19-kD glycoprotein is extrinsically bound to the external surface. WGA-

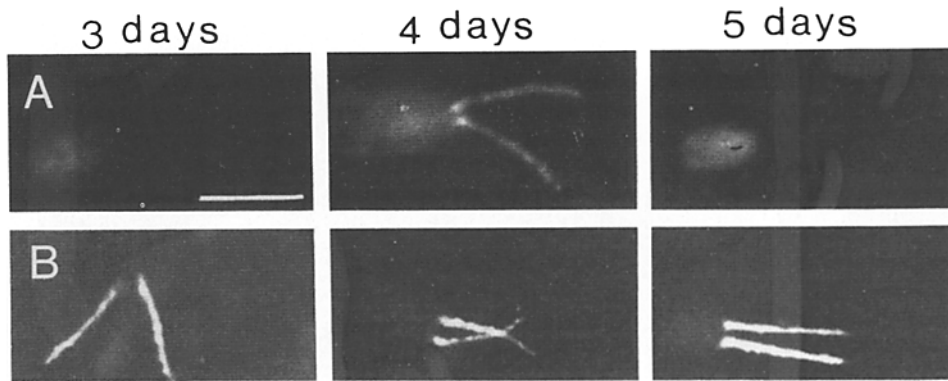


Figure 5. Gamete specificity of WGA binding to the flagellar surface of cells grown in liquid medium. Each day, the mating competence of the cells was determined as described by Tomson et al. (1985). 4 d after starting the culture, 30% of the cells were sexually competent. The next day the competence had returned to and was maintained at zero. Samples were also tested for their ability to bind WGA (A) or mAb 44.2 (B). Photographs were taken using equal exposure times for cells from different days. Cell body fluorescence was diminished using a filter (Kp 560; Carl Zeiss, Inc.). Bar, 10 μ m.

binding proteins were detected in a similar manner on the mt^- cell bodies of flagella-less cells. Five major labeled bands were found as shown in Fig. 6 C, lane 2. None of these components were detected on flagella nor were the 19- and 123-kD flagellar glycoproteins found in the cell bodies. Fur-

thermore, the soluble cell wall glycoproteins did not bind WGA (Fig. 6 C, lane 4). The spatial separation of flagellar and cell body membrane domains can be explained by the presence of a barrier in the transition zone at the bases of each flagellum (Musgrave et al., 1986).

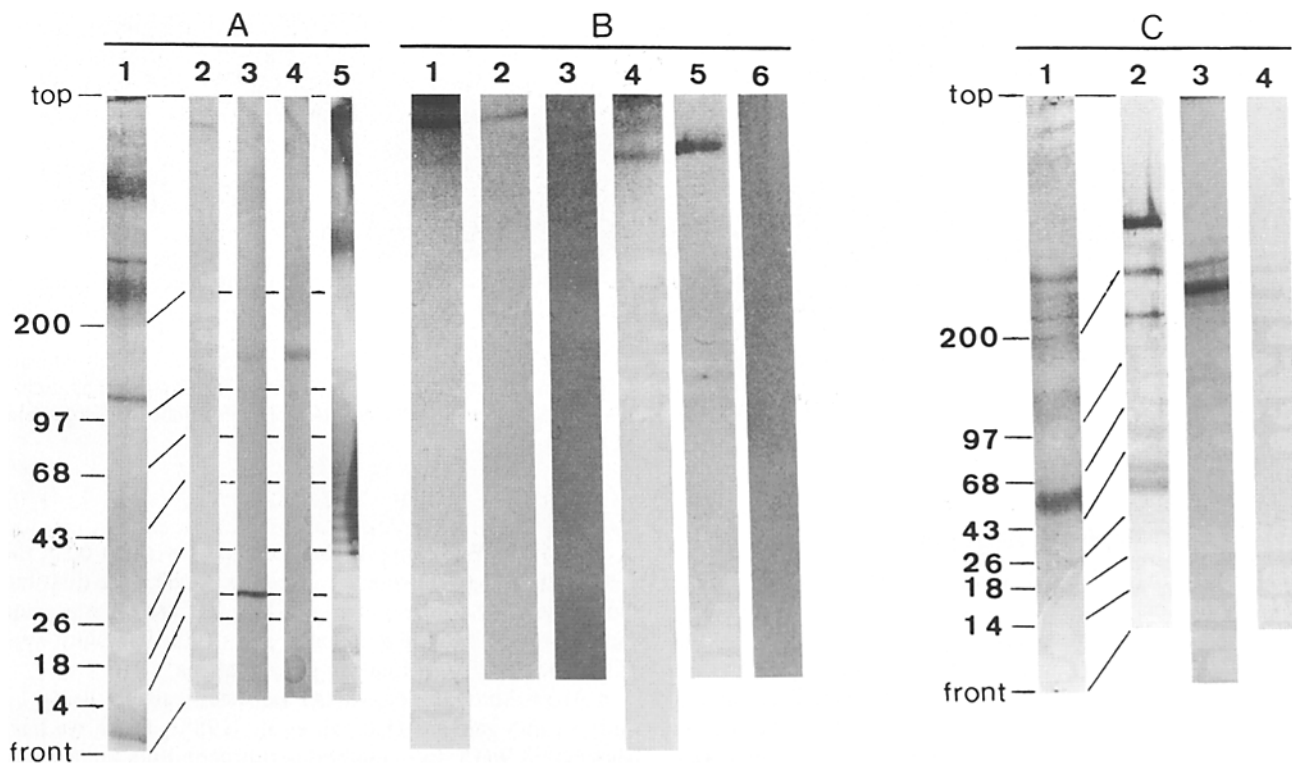


Figure 6. Gel electrophoresis and Western blot analyses. (A) Binding specificity of mAb 66.3, WGA, and mAb 44.2 to components in Triton X-100 extracts of flagellar vesicles obtained from strain 5.39.4: (lane 1) the glycoprotein composition after staining the gel with periodic acid Schiff reagent; (lane 2) a Western blot using mAb 66.3; (lanes 3 and 4) the components recognized by WGA in vesicles obtained from different batches of gametes; (lane 5) the major flagellar glycoproteins that are recognized by mAb 44.2. (B) Electrophoresis gels and Western blots of concentrated purified mt^- (lanes 1-3) and mt^+ (lanes 4-6) agglutinin. The gels were silver stained (lanes 1 and 4) according to Morrissey (1981). The binding of mAb 66.3 (lanes 2 and 5) and WGA (lanes 3 and 6) were visualized by immunostaining. (C) Binding of WGA to cell body glycoproteins extracted into Triton X-100 from strain 5.39.4. The extract was subjected to electrophoresis and stained with periodic acid Schiff reagent (lane 1) or transferred to nitrocellulose and stained with WGA (lane 2). Cell walls were isolated from the cell bodies, dissolved in sample buffer, and also subjected to electrophoresis. The cell wall glycoproteins were stained with periodic acid Schiff reagent (lane 3) or transferred to nitrocellulose and stained with WGA (lane 4).

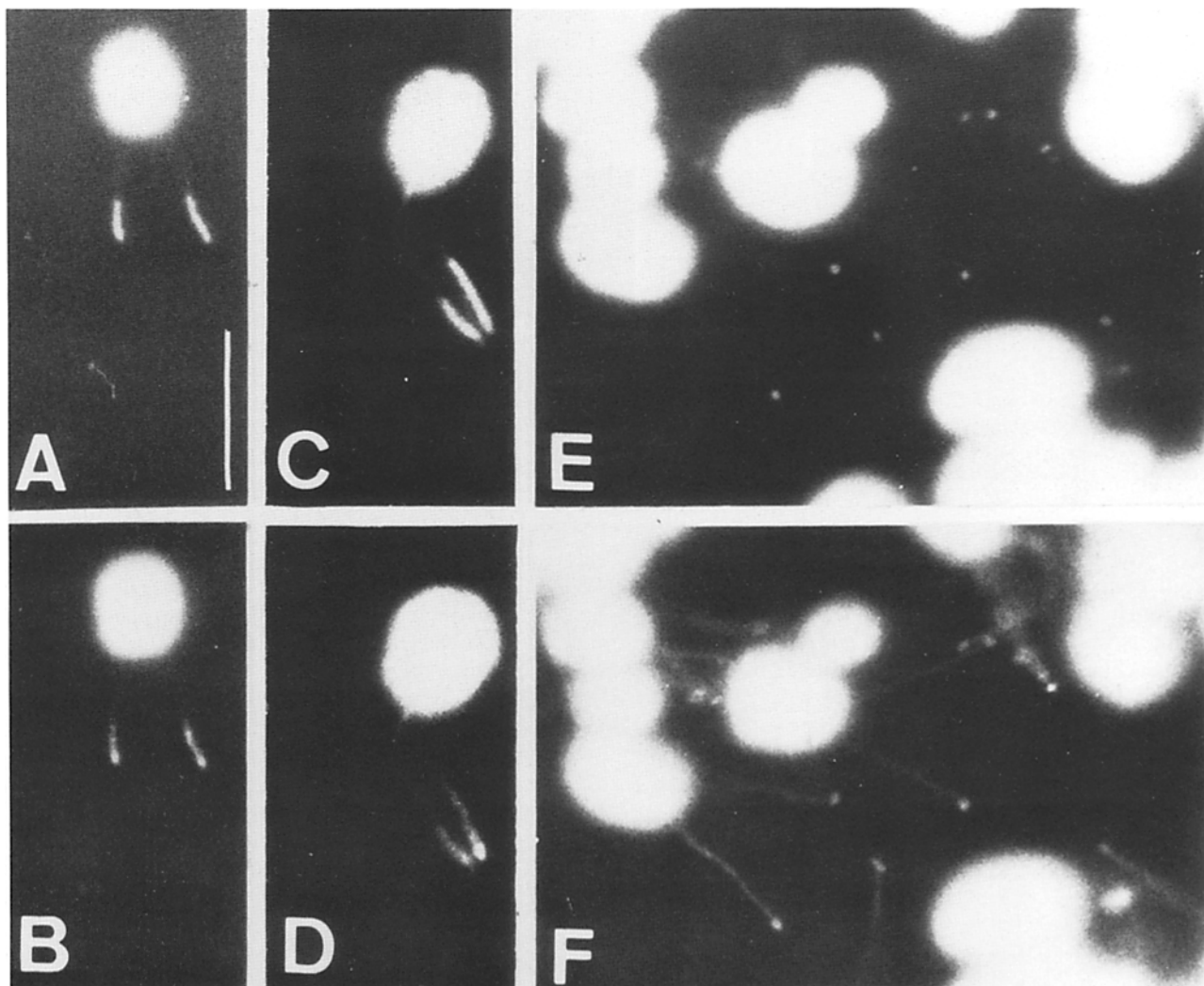


Figure 7. Double-labeling experiments showing the distributions of WGA-binding glycoproteins and agglutinin after tipping. Tipping was induced with 50 $\mu\text{g/ml}$ WGA (*A-D*) or with a 20 \times diluted solution of mAb 66.3 (*E* and *F*). After 30 min, the cells were fixed in glutaraldehyde. After removal of the fixative, the WGA-binding glycoproteins were labeled with WGA and TRITC-conjugated anti-WGA IgG, whereas the agglutinin was labeled with mAb 66.3 and FITC-conjugated goat anti-mouse IgG. FITC and TRITC were separately visualized using different filter sets. (*A* and *C*) Localization of WGA-binding glycoproteins after WGA-induced tipping; (*B* and *D*) localization of agglutinin after WGA-induced tipping; (*E*) localization of agglutinin after mAb 66.3-induced tipping; (*F*) localization of WGA-binding glycoproteins after mAb 66.3-induced tipping. Bar, 10 μm .

WGA-induced Tipping of Agglutinins

With the aid of mAb 66.3, it was possible to observe the redistribution of agglutinins during WGA-induced tipping. In double-labeling experiments, TRITC-conjugated anti-WGA and FITC-conjugated anti-mouse were used to visualize their respective antigens. As illustrated in Fig. 7, *A* and *B*, WGA-induced tipping of the WGA-binding glycoproteins was accompanied by tipping of the agglutinin. In this case, the distribution pattern of the agglutinin was very similar to that of WGA: long, drawn out tips of accumulated fluorescent material. This was often the case, but sometimes the agglutinins, as compared with the WGA-binding glycoproteins, were more concentrated at the flagellar tips. Intermediate situations, where the agglutinin was distributed as a gradient within the long tip of WGA-binding glycoproteins, were also found. The agglutinin concentration was then always highest at the tip, decreasing towards the base of the WGA zone (Fig.

7, *C* and *D*). When tipping was induced by mAb 66.3, the WGA-binding glycoproteins were also tipped. The distribution patterns of both components (Fig. 7, *E* and *F*) was again similar, except that the more basal parts of the flagellum were not cleared of WGA-binding glycoproteins.

mAb 66.3 has been shown to bind specifically to the agglutinin in mt^- gametes (Homan et al., 1988), while we have shown that WGA does not bind to this agglutinin but to other flagellar glycoproteins. Thus, these results indicate that mt^- agglutinin in the flagellar membrane is associated with one or more of these WGA-binding glycoproteins. Similar cotipping was also observed in mt^+ cells. However, it should be noted that, in mt^+ gametes, mAb 66.3 not only binds the agglutinin but also another flagellar component. Neither of them bound WGA. Cotipping is a rather specific process because the major glycoproteins of mt^- , recognized by mAb 44.2, were not redistributed after addition of mAb 66.3 or

WGA. Vice versa, mAb 44.2 treatment of live gametes did not invoke any redistribution of mAb 66.3- or WGA-binding glycoproteins or the major flagellar glycoproteins (data not shown).

Discussion

Recognition and adhesion between flagellar surfaces of mating *C. eugametos* gametes are mediated by mt^+ and mt^- agglutinins. No other components have been found to agglutinate cells of the other mating type (Musgrave et al., 1981; Klis et al., 1985). Furthermore, agglutination can be prevented by mAb 66.3 because it blocks the mt^- binding site (Homan et al., 1988). This indicates that a unipolar binding system is in operation. Nonetheless, other flagellar components must be indirectly involved in agglutination. For example, given the signaling function of agglutination, the agglutinins must be coupled to signal emitters and, since agglutinins can be redistributed over the flagellar membrane during mating (Homan et al., 1987), they can also be coupled to a contractile cytoskeleton. Since the signal emitters and the cytoskeleton are intracellular and the agglutinins are extracellular, we assume that they are coupled by an intrinsic membrane protein that anchors the agglutinins to the flagellar surface. In this way, we are forced to view each agglutinin as a member of a protein complex that is characteristic of gamete flagellar membranes.

To understand how agglutination exercises its multiple functions, it is necessary to identify the other members of the complex. In this respect, WGA could prove to be a useful tool. The lectin has the same effect on *C. eugametos* gametes as isoagglutinins, isolated flagella of the opposite mating type, or the mAb 66.3; it induces all mating reactions including the formation of mating structures and tipping of agglutinins. In addition, it elicits an increase in cAMP, which is an important signal formed during sexual agglutination (Pijst et al., 1984; Pasquale and Goodenough, 1987). This could be explained by assuming that WGA binds to the agglutinins, but it does not. It does not stain agglutinins on Western blots nor do agglutinins bind to Sepharose-WGA (data not presented). However, other flagellar components are labeled in the Western blots, and WGA clearly binds to NH_4Cl cells which do not possess agglutinins.

If WGA does not bind the agglutinins yet induces their tipping and all the mating reactions, then the simplest explanation for this activity is that it binds to another member of the postulated agglutinin complex in the flagella membrane. Being tetravalent, WGA then cross-links the complexes and, in doing so, triggers all subsequent reactions. In this postulate, the sexual binding site is not important for signal transduction. This is supported by the fact that WGA was able to induce the early mating responses in cells that could not agglutinate: for example, light-sensitive cells in the dark. Even NH_4Cl cells that did not contain any agglutinins were able to respond to WGA. Evidence in the literature supports this postulate: for example, Homan et al. (1988) showed that monoclonal antibodies that bound the agglutinin outside the sexual binding site (e.g., mAb 66.6) were just as effective as mAb 66.3 in inducing mating responses in live gametes. Second, it has been shown by Claes (1977) and Goodenough and Jurivich (1978) that polyvalent antisera raised against vegetative flagella of *C. reinhardtii* were effective surrogate aggluti-

nins when added to gametes or mutants lacking agglutinins (Claes, 1977; Goodenough and Jurivich, 1978; Goodenough et al., 1978; Hwang et al., 1981; Adair et al., 1983). Agglutinins should therefore be seen as members of a protein complex that bind their counterparts on partner flagella and thus cause their aggregation. This cross-linking of complexes seems to be essential because Claes (1977) and Goodenough and Jurivich (1978), using *C. reinhardtii*, and Homan et al. (1988), using *C. eugametos*, reported that monovalent Fab fragments were biologically inactive.

We have not identified the WGA-binding glycoprotein that is complexed with the agglutinins, but only two major candidates appear from our results: the 19- and 123-kD glycoproteins. However, it is unlikely that both WGA-binding glycoproteins are present in the complex; although WGA-binding glycoproteins cotip with the agglutinins, the association is not absolute. Thus, mAb 66.3 induces tipping of all the agglutinins but only some of the WGA-binding glycoproteins, the rest being evenly distributed over the whole flagellum. Vice versa, while WGA induces tipping of all WGA-binding glycoproteins and all agglutinins, the agglutinins are sometimes more concentrated at the flagellar tips than the WGA-binding glycoprotein. These results are best explained by assuming that only one WGA-binding protein is complexed with the agglutinin. Alternatively, the agglutinins may not be complexed at all, but WGA may induce a signal that triggers tipping of the agglutinins and some other components. However, we do not favor this possibility because, during sexual agglutination, not all the agglutinins are tipped (Homan et al., 1988) as this alternative model suggests.

Although we tend to discriminate between agglutinin-complexed WGA-binding proteins and the others based on our wish to understand the function of the agglutinins, it should be emphasized that both types are of interest because both can be redistributed over the flagellum and are gamete specific. In general, flagellar glycoproteins do not have these properties and the major glycoproteins recognized by mAb 44.2 have never been seen to change in distribution under any circumstances. We assume therefore that they are permanently coupled to an immotile cytoskeleton or at least that they cannot be coupled to the contractile system as can the agglutinin complex. This is in contrast with the situation for *C. reinhardtii*, where the dominant flagellar glycoprotein can be transported over the flagellar surface (Bloodgood and May, 1982; Bloodgood et al., 1986).

This difference in protein motility may explain another discrepancy between the two species. *C. reinhardtii* gametes can be sexually activated by relatively nonspecific agents such as polystyrene, concanavalin A, and polyclonal antisera against vegetative flagella (Claes, 1977; Goodenough and Jurivich, 1978). We are not aware of equivalent reactions in *C. eugametos*; while general cross-linkers (such as concanavalin A [Musgrave et al., 1979b], polyclonal antisera against flagellar proteins [Lens et al., 1980], or even mAb 44.2 that binds an epitope present on several major glycoproteins [Homan et al., 1987]) have been used, they have not been seen to activate gametes. In the case of mAb 44.2, we know that it does not bind the agglutinin nor the WGA-binding proteins reported here and thus may not be able to cluster the agglutinin complex; it seems likely, however, that the other two agents can cross-link at least one of the components in this complex. Nonetheless, there is no biological

effect and, even when the cells are isoagglutinated, they lack the vibrating appearance of sexual agglutination. Only anti-agglutinin antibodies, such as mAb 66.3 (Homan et al., 1988) and now WGA, that cross-link a limited number of glycoproteins, including the agglutinins, sexually activate *C. eugametos* gametes. This could reflect the difference in motility of the major flagellar glycoproteins in the two species. In *C. reinhardtii*, most glycoproteins are motile in the membrane. Thus, an agent that cross-links an agglutinin with another glycoprotein may limit its motility but still permit some clustering of agglutinins. In *C. eugametos*, such cross-linking could immobilize the agglutinin and prevent clustering and signal transduction.

WGA induced a sexual response in cells lacking active agglutinin but did not induce the formation of papillae. Thus, while the agglutinin seems to be expendable for the induction of the early mating responses, it seems necessary in an active form to trigger the protrusion of the papilla. These results indicate that both the WGA-binding component and agglutinin may be needed for optimal signal transduction. This possibility will be investigated by measuring cAMP responses in the presence and absence of agglutinin.

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